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AN IMPROVED METHOD FOR THE PURIFICATION OF CHLOROPHYLLS *A* AND *B;* QUANTITATIVE MEASUREMENT OF THEIR ABSORPTION SPECTRA; EVIDENCE FOR THE EXISTENCE OF A THIRD COMPONENT OF CHLOROPHYLL

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(with plate X and three figures)

Introduction

The existence of two components of chlorophyll was first announced as a logical deduction from experimental data by Stokes (**8**) in 1864. He used the method of fractionation between solvents (**9**), carbon bisulphide and ethyl alcohol in his case, to separate the two green components of chlorophyll from each other and from the yellow pigments, carotene and xanthophyll, all of which he differentiated spectroscopically. The existence of two components of chlorophyll was concluded incorrectly two years earlier, in 1862, by Simmler (**5**) from his observation that chlorophyll fluoresced in both the red and the green regions of the spectrum. This same observation had been made in 1852 by Stokes (**7**), who studied the fluorescence of alcoholic leaf extracts visually through a prism. Sorby (**6**), using fractionation methods with carbon bisulphide and aqueous ethyl alcohol, distinguished between three different chlorophylls: (1) blue chlorophyll which formed a bluish green solution, (2) yellow chlorophyll which formed a yellowish green solution, and (3) chlorofucin.

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His methods, although not clearly described, may be criticized severely because they involve treatment of the chlorophyll preparation with heat, alkali, and perhaps impure solvents. Moreover, he used algae in much of his work and in no single group of algae (brown, red, or green) did he find all three components.

A new method of chlorophyll component separation was introduced by TSWETT (10) in which a petroleum ether, benzene, or carbon bisulphide solution of crude chlorophyll was filtered through a column of calcium carbonate or sugar. He states that carbon bisulphide is the most efficient. In this way a chromatogram was obtained, and the different pigments were found separated in zones of the adsorbent. These zones were separately extracted with suitable solvents and solutions of the various pigments were thus obtained. In addition to a number of xanthophylls in various yellow zones, TSWETT found two chlorophyll pigments in adjacent zones. The upper zone was dark olive green in color and contained chlorophyllin β; the lower one was dark blue-green and contained chlorophyllin α. They correspond to the chlorophylls *b* and *a* of WILLSTÄTTER, whose work will be considered later. TSWETT (11) studied these green pigments spectroscopically with the methods then available.

Thus far, neither the chlorophyll components nor their mixture had been prepared in a state free from colorless impurities and yellow pigments. Chlorophyll was isolated from such impurities by WILLSTÄTTER *et al.* (13) in a pure and unchanged state and the components were supposedly separated from each other, but, as will be shown later, this separation was incomplete. By making certain additions to their method, components *a* and *b* have been completely separated from each other and isolated in the pure state. Evidence will be presented of the possibility of the existence of a third component.

Throughout this discussion, the term chlorophyll refers to the green (and blue) fat-soluble pigments extracted from fresh green plant tissue, and is not to be confused with "la chlorophyll naturelle" of LUBIMENKO (3) or the "phyllochlorins" of MESTRE (4). The latter refer to the *in vivo* green pigments composed of the chlorophyll components plus whatever proteins, lipoids, or yellow pigments which may be in combination with them in the living chloroplast.

Experimentation

In the present investigation the preferential adsorption method of TSWETT was combined with WILLSTÄTTER'S method of fractionation between immiscible solvents to isolate chlorophyll *a* in a pure state, free from component *b*. A fractional precipitation method was used to prepare component *b* free from *a*. Inert and specially purified solvents were used throughout, no chemical reactions were employed, and the chlorophyll was never heated above room temperature. The test of purity which is most reliable is the absorption spectrum of the component considered. This is true because of the great accuracy and precision with which the absorption of light at any desired wave length may be measured by use of the spectro-photoelectric method (**16**) developed recently and applied to the chlorophyll pigments.

METHOD OF ISOLATION OF CHLOROPHYLL COMPONENTS *a* AND *b*

Following is a brief outline of the method used for the isolation of pure chlorophylls *a* and *b* from fresh leaf material. This method is based on results from fifty-five component separations and is the shortest method found by the writer to give the most efficient separation of the chlorophyll components. That part of the method above the heavy line of the chart follows the procedure of WILLSTÄTTER and STOLL very closely, although some variations are introduced which are important to secure a complete isolation of *a* and *b*. It must be made clear that if WILLSTÄTTER'S procedure is followed exactly, especially in regard to certain details, one obtains the same results as his, and the preparations have the same absorption spectra as those reported by WILLSTÄTTER and STOLL (**13**). Since their method may be extended and modified to produce components with very different absorption spectra, separations were made under widely different conditions and many steps of the procedure were varied in order to determine whether or not these new results were due to experimental error.

The steps of the method are discussed in detail as follows. The percentage composition of all solvents used in the preparative procedure is expressed on the basis of volume unless specifically stated otherwise.

METHOD OF ISOLATION OF CHLOROPHYLL COMPONENTS *A* AND *B**

Ground fresh leaves
(Extract with acetone)
I↓
Acetone extract
(Add to petroleum ether and wash with H_2O, 80% acetone, and H_2O)
II ←→ IIa

Petroleum ether layer containing chlorophyll, carotene, and xanthophyll (Wash with 80% CH_3OH)

Aqueous layer containing acetone, water-soluble pigments (as anthoxanthins) and colorless impurities

III ←→ IIIa

80% CH_3OH containing xanthophyll

Petroleum ether solution containing chlorophyll, carotene, and some xanthophyll
(Wash with H_2O)

IVa ←→ IV

Petroleum ether suspension of *a*; carotene and xanthophyll in solution (Filter on talc and extract with ether)

H_2O+CH_3OH

V ←→ Va

Petroleum ether solution of carotene and xanthophyll

Chlorophyll (*a*+*b*)+small amounts of xanthophyll and carotene
(Transfer to petroleum ether, wash with H_2O, 85%, and 90% CH_3OH)

VI ←→ VII

Petroleum ether solution rich in *a* (Wash with H_2O)

CH_3OH solution rich in *b* (Wash with petroleum ether)

↓VI 1 ←→ ↓VII 1 ←→

H_2O+CH_3OH

Petroleum ether suspension rich in *a* (Filter on talc and extract with ether)

CH_3OH solution rich in *b* (Transfer to ether; wash with H_2O)

Petroleum ether solution rich in *a*

↓VI 2 ↓VII 2 ←→

Ether solution rich in *a*

Ether solution rich in *b* (Add to petroleum ether and filter on talc)

H_2O+CH_3OH

(Add to petroleum ether)

↓VI 3† ↓VII 3 ←→

Ether-petroleum ether solution rich in *a* (Filter on thick talc filter) Last traces of yellow pigments removed in filtrate

Component *b*, precipitated on talc (Extract with ether and filter)

Petroleum ether solution of *a*+*b* +last traces of xanthophyll

↓VI 4 ↓VII 4

Green and *blue* zones (Separate mechanically)

Ether solution of *b*

| VI 5

←→

Green talc with adsorbed *a*+(*b* or "c"?) (Extract with ether)

Blue talc with adsorbed *a* (Extract with ether)

↓VI 6

Green ether solution of mixture of *a*+(*b* or "c"?)

↓VI 7

Blue ether solution of *a*

* The distribution of the yellow pigments between solvents was obtained by private communication with ELMER S. MILLER.

† Steps VI 3–7 are additions to the procedure of WILLSTÄTTER and STOLL.

STEPS OF PURIFICATION METHOD

I. From 1.0 to 1.5 kg. of fresh green leaves are ground rather coarsely with a food grinder and collected in an enameled pan. Within two or three minutes after the grinding process, the juicy pulp is treated with 500 cc. of acetone and thoroughly stirred in a Pyrex beaker. A 1.5 ft. square of washed muslin is spread over the enameled pan and the mixture is placed on this cloth. As much as possible of the liquid is squeezed out by hand. More of the liquid is then extracted by the use of a hand press, lined with tin. The pulp is again extracted in the same fashion with 500 cc. of acetone. Most of the water is removed by these two extractions, the first of which is yellow and the second yellowish green. They contain very little chlorophyll and a large amount of yellow anthoxanthins and colorless substances, and are therefore discarded.

The press cake from the second extraction is reground and the meal is then treated with 400 cc. of acetone, stirred, and placed in the cloth. As in the first treatment, the liquid is removed from the plant meal, first by hand and later by the press. The deep green extract must be filtered by suction to remove solid particles of plant meal. The extraction process is repeated several times. The third, fourth, and fifth extracts contain most of the chlorophyll. These acetone solutions are saturated with chlorophyll and some of it usually precipitates on the filter paper with the solid sediment of leaf tissue particles. When this occurs, 25 to 50 cc. of acetone may be poured through the filter to remove the chlorophyll. A total of about eight extractions is usually necessary to remove the chlorophyll to such an extent that the meal becomes gray and the extract very light green.

II. The acetone extracts are filtered separately and added to a mixture of 1.0 liter of petroleum ether (b.p. 35–55° C.) and 100 cc. of acetone in a 4-liter glass separatory funnel. The extracts are poured through a small funnel in such a manner that they flow slowly down the side of the separatory funnel. After the first extract has been added and the contents of the separatory funnel have been rotated gently, some chlorophyll remains in the acetone-water layer, which is drained off and discarded. Care must be taken that the mixture is not too vigorously agitated, especially at this point, in order that emulsion formation may be avoided. After the second addition of extract to the petroleum ether, the added acetone usually mixes easily with the petroleum ether and forms a clear solution that is bright red by transmitted light. The volume of the acetone-petroleum ether mixture increases to 2.5–3.0 liters by addition of the extracts.

A small electric filament lamp is convenient as a means of examination of solutions and suspensions throughout the whole separation process.

The lamp is held close to the solution on the side of the container opposite the eye. The nature of the appearance of the filament is a good indicator of the chlorophyll's condition in the liquid. When the filament appears red and well defined, the chlorophyll is in true solution and no emulsion is present. An emulsion has a cloudy appearance. Precipitation of the chlorophyll causes disappearance of the red color and formation of black particles and the suspension becomes opaque.

IIa. While the mixture is rotating, water is run gently down the sides of the funnel from a second funnel above the large one. Most of the acetone goes into the aqueous layer, accompanied by colorless and some yellowish impurities. When about 2 liters of water have been used in this manner, emulsions begin to form and the washing with water is discontinued. The petroleum ether layer is then washed twice with 150 cc. of 80% acetone and again with water until emulsions start to form.

III. After most of the acetone has been washed from the petroleum ether layer with water, the petroleum ether solution of chlorophyll, carotene, and xanthophyll is washed five times with 200 cc. of 80% methyl alcohol to remove most of the xanthophyll. The first two of these methyl alcohol washings are dark green in color, the last one light greenish yellow.

IIIa. The petroleum ether solution of chlorophyll is later to be washed with 85% methyl alcohol as part of the separation of *a* and *b*. Since the carotene is present, emulsions form rather easily with 85% methyl alcohol; therefore it is desirable to remove the carotene at this point.

IV. The petroleum ether solution of chlorophyll, carotene, and 15–20% of original xanthophyll is washed cautiously with water, which removes the methyl alcohol.

IVa. The chlorophyll precipitates when all of the methyl alcohol is removed. The particles can be seen with the aid of the electric lamp; the lamp filament loses its red color when seen through the suspension. When complete precipitation occurs, the fluorescence disappears and the suspension becomes very opaque to light. The presence of yellow pigments makes complete precipitation difficult.

V. The suspension, after drying over anhydrous sodium sulphate, is filtered through a layer of talc about 3.0 cm. in thickness. This talc filter is made on a Buchner funnel of 15 cm. diameter and 5.5 cm. depth. The talc layer is made by wetting the talc with petroleum ether and applying gentle suction. The talc is pressed down with a large spatula while suction drains off the petroleum ether until the talc forms a hard and fairly dry layer. The precipitated chlorophyll forms a thin black layer on top, which needs slight stirring occasionally to facilitate filtration. Usually the

chlorophyll is not completely precipitated and some of it penetrates the talc a short distance. Several washings with petroleum ether remove all but a trace of the carotene from the top black layer. Some xanthophyll precipitates with the chlorophyll.

Va. After the black layer of chlorophyll has become fairly dry by suction, it is scraped off with a spatula and immediately placed in anhydrous ether.[2] A complete stirring of this mixture insures solution of all of the chlorophyll in the ether. The talc is removed by filtration and an ether solution of chlorophyll plus some xanthophyll and a trace of carotene results. This is evaporated quickly to dryness by means of an electric fan, and heat is thus avoided.

VI–VII. The chlorophyll is dissolved in as small a volume of ether as possible and added to 500 cc. of fresh petroleum ether. Two washings with 200 cc. each of 80% methyl alcohol remove most of the ether and are discarded. The petroleum ether solution is then extracted twenty times, each time with 500 cc. of 85% methyl alcohol. Finally, three extractions with 500 cc. of 90% methyl alcohol are made. Before use, the methyl alcohol solutions are saturated with petroleum ether, and to prevent allomerization, 0.01 gm. of oxalic acid per liter is added. After each methyl alcohol addition has been made the whole mixture is rotated gently to provide more complete contact between the two phases. According to WILLSTÄTTER and STOLL, who extracted only sixteen times with 85% methyl alcohol, these washings remove most of component *b* from the petroleum ether layer and the 90% methyl alcoholic washings extract the last traces of *b*. The petroleum ether solution is rich in chlorophyll *a* and usually contains a small amount of yellow pigments.

VI 1. This petroleum ether solution is washed with water until the methyl alcohol has been removed and complete precipitation occurs. Thick layers of the mixture are now opaque and particles of chlorophyll are easily visible. All fluorescence has disappeared and the suspension is black.

VI 2. After drying with sodium sulphate, the suspension is filtered on a 2 cm. layer of talc and the precipitated chlorophyll is washed several times with petroleum ether. The top black layer is mechanically removed and extracted with ether. If a small amount of the chlorophyll penetrated the talc this may be discarded, as the amount lost will be small.

VI 3. The green ether solution is evaporated to dryness by an air current. The water of condensation may be easily poured off when the ether has completely evaporated.

At this point, the method of WILLSTÄTTER and STOLL ends, this prepa-

[2] Ether signifies di-ethyl ether unless specifically designated petroleum ether.

ration being designated as component *a*. Further to purify this preparation, the pigment is dissolved in 70 cc. of ether and added to 300 cc. of petroleum ether (b.p. 35–55° C.). No chlorophyll is precipitated and a bluish green solution results.

VI 4. This intensely fluorescent solution is filtered through a thick talc filter, 4.5 cm. thick, made as described in step V. No precipitated layer forms on top of the filter and the chlorophyll penetrates the talc, forming a chromatogram (pl. X, B). After two washings with petroleum ether, the talc is moderately dried by suction and gradually removed from the top with a spatula. The last traces of yellow pigments are removed in the filtrate.

VI 5. A green zone (pl. X, B 1) is found in the top portion of the talc layer. The upper fifth of this green zone is lighter in color than the remainder. Below the green zone is a *blue* zone (pl. X, B 2). The thickness of the green layer is from one-third to one-half that of the total colored talc layer. If the thickness of the talc is not sufficient to adsorb the amount of chlorophyll present in the solution, some of the blue fraction will be found in the last portion of the filtrate. Provided the solution has been properly prepared in regard to ether concentration, there is a sharp line of demarcation between the green and blue zones. Should the ether concentration be too high, however, a good separation of the blue from the green pigments is not obtained and the chlorophylls will not be adsorbed on the talc; if it is too low, the color zones will be too thin to permit efficient separation or some chlorophyll will precipitate as a black deposit on top of the talc. To obtain these two regions of talc uncontaminated with each other, a middle section, which includes the line of demarcation, is discarded. With care this discarded section may be made quite small. It is especially necessary that the blue layer be uncontaminated with the green.

VI 6, 7. The separated portions of talc are immediately placed in anhydrous ether, thoroughly stirred, and filtered quickly. The chlorophyll is removed as quickly as possible from the talc with fresh ether and the washed talc is discarded. From 300 to 400 cc. of ether is required for each talc portion. The solutions are evaporated to dryness by an air current. Water of condensation collects beneath the chlorophyll, owing to the rapid evaporation of ether. This water layer prevents the chlorophyll's adherence to the glass, which in turn facilitates the removal and pulverization of the pigment.

The green solution contains component *a* plus one or more other components. These will be considered later in more detail. The blue ether so-

lution, if sufficiently concentrated, is pure *blue* by transmitted light and contains pure chlorophyll *a*.

Purification of component *b*

VII 1. The 85% methyl alcohol extracts are washed with petroleum ether to remove chlorophyll *a*. To each of the first five extracts, 120 cc. of methyl alcohol is added to raise the concentration to 90%. After the fifth extract the methyl alcohol addition is decreased 12 cc. each time. These extracts are added in pairs to 120 cc. of petroleum ether and washed by gentle rotation to remove *a*, a large part of which goes to the petroleum ether layer. The carotene is completely removed by the petroleum ether. The twenty 85% methyl alcohol extracts are divided into three groups: (1) extracts 1 to 4; (2) extracts 5 to 10; and (3) extracts 11 to 18. The remaining two 85% methyl alcohol extracts contain too little chlorophyll *b* for recovery and the 90% methyl alcohol extracts contain too much chlorophyll *a* for efficient isolation of *b*.

VII 2. The groups of extracts are mixed with 500 cc. lots of ether and sufficient water is added to cause a separation into two phases. The aqueous layer, which is light yellow or colorless, is discarded. The ether solutions are washed with water to remove methyl alcohol and are then evaporated to dryness with an air current.

VII 3. These preparations of component *b* are each dissolved in 13 cc. of ether, and 100 cc. of low-boiling petroleum ether (b.p. 30–35° C.) is added. The result is a true solution (red by light transmitted through thick layers and no particles visible) and remains clear for about 15 minutes if allowed to stand until part of the ether has evaporated. An air current will hasten the precipitation of chlorophyll *b*, which may be closely followed by observing the formation of precipitated particles. When approximately two-thirds of the total pigment (estimated visually by intensity of transmitted light) has precipitated, the suspension is filtered on a thin layer of well compressed talc in a small Buchner funnel. The precipitated portion, consisting of chlorophyll *b*, is carefully removed from the filter, including as little talc as possible. The dissolved portion contains xanthophyll, component *a*, and some *b*, which penetrate the talc filter and are discarded.

VII 4. The precipitated *b* is dissolved from the talc with ether. The solution is filtered and evaporated to dryness with an air current. Of the three resulting preparations of *b*, only the third (extracts 11 to 18) is usually pure. When the phase test (saponification of an ether solution with methyl alcoholic potassium hydroxide) is applied, and water is added after

complete saponification, the ether layer is colorless if all xanthophyll and carotene have been removed. In the case of *b* from extract group 3, the ether layer is colorless, but that from group 2 is usually slightly yellow and that from group 1 is distinctly yellow. If the chlorophyll from groups 1 and 2 were reprecipitated by the preceding method, probably the xanthophyll could be completely removed. Since this reprecipitation is very wasteful of *b*, larger quantities should be used than may be obtained from 1 kg. of fresh leaves.

Discussion of chlorophyll chromatograms

Evidence from more than one type of data points toward the existence, in an acetone extract from fresh green leaves, of more than two components of chlorophyll. The green component which is called "component *c*" in this paper, and is found in the green layer ("fraction c") of the talc in step VI 4, may be a third component of chlorophyll. Evidence will be presented here and later in the paper that component *c* has some properties similar to *a* and others similar to *b*. In the green fraction c there may be more components than component *c*, in addition to *a*.

Some chlorophyll was extracted with acetone from fresh grass, transferred to 1 liter of petroleum ether, and washed five times with 450 cc. of 75% methyl alcohol to remove most of the xanthophyll. After precipitation by washing with water, ether was added until the fluorescence reappeared. This mixture, containing *a*, *b*, component *c*, carotene, and doubtless some xanthophyll and colorless impurities, was filtered on talc in a glass Gooch funnel. The chromatogram (pl. X, A) was washed with fresh petroleum ether to remove carotene and perhaps part of the xanthophyll.

Four distinctly different zones of approximately equal thickness were found in the talc. They were separately extracted with ether and the phase test was applied to each solution. The upper zone, 1, was very light grayish green and contained mostly colorless impurities with only a small amount of chlorophyll. The very thin darker green layer of zone 1 was not isolated, but was discarded with the remainder of zone 1. The ether solution (from zone 1) gave a yellowish brown phase test. Zone 2, dark green in color, gave a brown phase. Zone 3 was lighter green than the second. Zone 4 was pure *blue* and contained component *a*. The ether solutions of these lower

two zones gave yellow phases. These phase tests indicated that *b* was present mostly in the dark green zone 2, mixed with *a*. Of the four zones, only the lower one, zone 4, contained a pure substance, as determined by color and phase test.

The ether solutions from the zones formed in step VI 4 give yellow phase tests. When the ether-petroleum ether solution of the green and blue pigments ((VI 3) is filtered through the talc, the green is adsorbed in a layer above the blue (pl. X, B). As more solution is filtered through the talc these colored zones widen. The blue layer always precedes the green layer. Probably two processes occur during the filtration: (1) the green displaces the blue, which is readsorbed lower down in the filter; (2) the green is adsorbed upon or beside the adsorbed blue on the talc surface but does not cover all of the adsorbed blue because the ratio of green to blue is too small. When the green layer (pl. X, B 1) is extracted separately with ether and the filtration of step VI 4 repeated, a blue zone is formed which is much thinner in comparison with the green zone than was the case in the first filtration. A third filtration of the green fraction produces a blue zone having a depth equal to only about 5% of the total.

The separation of the blue and green fractions by differential adsorption on talc is most efficient from an ether-petroleum ether solution which is red by transmitted light and strongly fluorescent. Twenty per cent ether (by volume) is the optimum concentration. When methyl alcohol is used instead of ether, and if exactly the proper amount of methyl alcohol is present, a good separation occurs. The separation is more difficult to regulate and control in this case, as a slight excess or insufficiency of methyl alcohol causes too little or too much adsorption in the talc to produce a good separation. The relative thickness of a zone is not an exact indication of the relative amount of chlorophyll adsorbed in that zone. The colors and contrasts of the zones fade rapidly upon drying.

Other solids were studied on a small scale as adsorptive agents for the separation of *a* and fraction c to determine to what extent the adsorptive surface might be specific for this separation. Stock materials were used and no special washings were applied for purification purposes. Powdered sucrose, powdered calcium carbonate (c.p.), and infusorial earth (fairly white) gave separations of the blue and green fractions equally as definite as those obtained with talc. Magnesium oxide was not so satisfactory, since the lower layer was blue-green and the upper one gray. Kaolin

(rather dark colored) adsorbed the chlorophyll more strongly than did the other solids used. The zones were thinner and their color differences were somewhat masked by the color of the kaolin.

Absorption spectra of components *a* and *b*

The absorption spectra of the chlorophyll components *a* and *b* in ether solution were studied quantitatively by a photoelectric method developed by ZSCHEILE, HOGNESS, and YOUNG (**16**). The resulting light absorption curves are presented in figure 1. Measurements were taken at intervals of 25 Å. The spectral regions isolated by the monochromator varied in width from 6 Å. at λ 3950 Å. to 13 Å. at λ 7000 Å. Solutions were changed after every sixth reading (at intervals of 150 Å.) to avoid photodecomposition of the chlorophyll.

Values of a, the light absorption coefficient defined by BEER's law,

$$I_x = I_o \times^{-acx}$$

$$a = \frac{\log \frac{I_o}{I_x}}{cx},$$

are plotted as ordinates of the curves. In this equation,

I_o = intensity of light transmitted by the solvent-filled cell.

I_x = intensity of light transmitted by the solution-filled cell.

x = thickness of absorption cell in cm. (4.25 cm.).

c = concentration of chlorophyll in gm. per liter.

The logarithm is to the base 10; a is expressed in liters per gram centimeter.

A concentration of 0.0007 gm./liter was used in the blue and red regions of the spectrum where absorption is greatest. In the green and yellow regions a concentration of 0.0150 gm./liter was used. The ether was freshly distilled over sodium and the solutions were kept in the dark. The values of a at the maxima and minima of absorption were accurately determined with solutions whose concentrations were known with an accuracy of 1.0%. The ratio $\frac{I_o}{I_x}$ varied between 1.5 and 3.0. These values of a at the maxima and minima were determined with a precision of better than 0.5%. a was calcu-

lated from BEER's law for all wave lengths measured, and when these values, derived from measurements at different concentrations, were

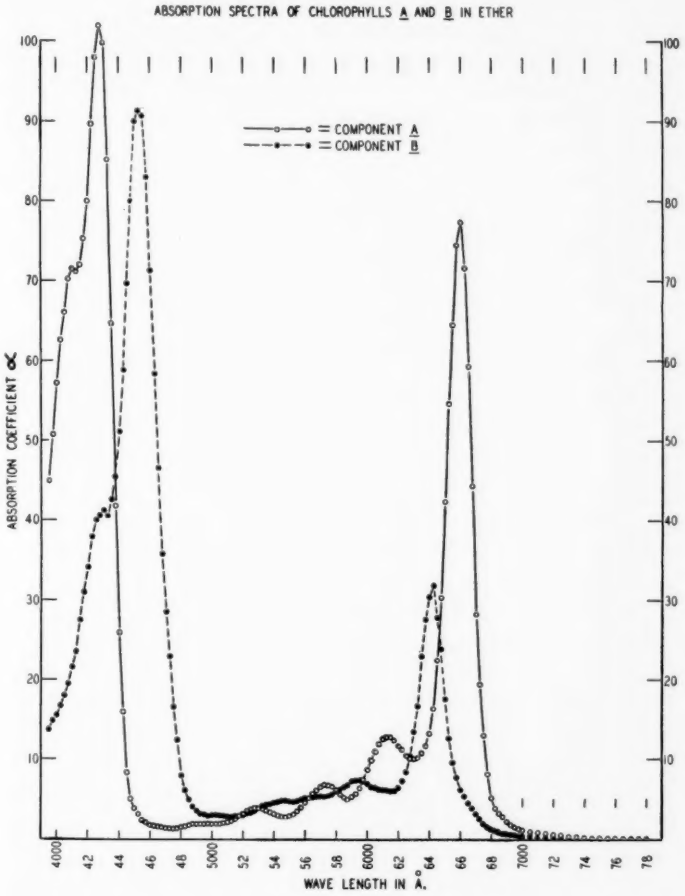

FIG. 1

plotted against wave lengths, it was found that the overlapping portions agreed very closely. This demonstrates that BEER's law is obeyed at these concentrations in ether solution. At the maxima and

minima the accuracy of a is 1.0% or better and at other points on the curves it is 2.0%. The wave lengths of the maxima are accurate to 10 Å. The values of a and λ for the maxima are presented in table I.

These absorption spectra of the chlorophyll components, prepared by the improved technique just described, will now be compared with those obtained by WILLSTÄTTER and STOLL (**13**, pls. VI, VIII). According to WILLSTÄTTER, the absorption spectra of the methyl chlorophyllides, as observed visually, are the same as those of the pure components.

TABLE I

ABSORPTION MAXIMA OF COMPONENTS *a* AND *b* IN ETHER SOLUTION (LISTED IN ORDER OF DECREASING INTENSITY)

CHLOROPHYLL *a*		CHLOROPHYLL *b*	
WAVE LENGTH IN Å.	*a*	WAVE LENGTH IN Å.	*a*
4275	102	4525	91.3
6600	77.4	4300	41.2
4100	71.5	6425	31.8
6125	12.7	5925	7.36
5725	6.75	5675	5.27
5275	3.86	5475	4.77
4975	1.93	5025	2.99

In the spectrum of *a* there is no trace of a band at λ 4500–4600 Å., such as WILLSTÄTTER reported. Since the chief absorption band of *b* has a maximum at λ 4525 Å., it would seem that WILLSTÄTTER'S *a* was contaminated with *b* or with some other substance which absorbs strongly in this region. The smaller band at λ 4100 Å. could not have been detected by WILLSTÄTTER'S method; otherwise the absorption bands of *a* have about the same order of intensity as that reported by WILLSTÄTTER.

In the spectrum of *b*, it is noted that the 6425 Å. band is much less intense than the 6600 Å. band of *a*. The 6425 Å. band has a steep slope toward the red side and shows no trace of a band at λ 6600 Å. WILLSTÄTTER'S *b* has an intense band at λ 6600 Å., which would be caused by an impurity of *a*. Also his *b* shows a weak band at λ 6150 Å. This would be caused by an impurity of *a*, which has a strong band at that same wave length. The 4300 Å. band of *b* is not caused

by an impurity of *a*, whose most intense band occurs at this wave length. This is evident from consideration of the relative intensities of the corresponding bands of the two components. Since no trace of a band at λ 6600 Å. is found in the spectrum of *b* (although *a* has a band at this wave length which is 2.5 times as intense as the 6425 Å. band of *b*), one would not expect *b* to have a band at λ 4300 Å. due entirely to an impurity of *a*, because the 4275 Å. band of *a* is not of much greater intensity than is the 4525 Å. band of *b*.

To obtain these absorption spectra curves, an accurate method was used which is far more sensitive to changes of light intensity than is the eye or photographic plate. It is evident that certain bands reported by WILLSTÄTTER and STOLL are not present. In the case of each component, these missing bands would be located in the spectral regions where the most intense bands of the other component are found. These data indicate that WILLSTÄTTER'S components were not pure, and that his separation of them was not complete. The series of seven absorption bands of components *a* and *b* in ether solution show certain similarities in relative intensities. The weaker bands of *b*, however, are not so well separated as are those of *a*. The general structures of the two spectra are the same, while those of WILLSTÄTTER and STOLL'S spectra are dissimilar in some respects.

TSWETT (**11**), who used the method of preferential adsorption on calcium carbonate to separate the chlorophyll components, reported the absorption spectra of these components, α and β, as observed visually with a microscope equipped with a spectral ocular. Although the color of TSWETT'S component *a* in ether solution was described as a pure beautiful blue, his preparations probably contained some component *b*, carotene, or xanthophyll, because his solutions of greatest thickness showed absorption at λ 4700 Å. appearing as a shadow before the terminal absorption region. TSWETT'S *b* evidently contained some *a* as an impurity, since its absorption spectrum had a band at λ 6100–6150 Å., where *a* absorbs strongly. In the spectrum of *b*, TSWETT did not find bands at λ 6600 Å. where *a* absorbs or at λ 5025 Å. where *b* absorbs. DHÉRÉ and ROGOWSKI (**1**) prepared the chlorophyll components by TSWETT'S adsorption method, and found that they had the same visible absorption spectra as those given by WILLSTÄTTER. They describe component *a* as pure blue.

GHOSH and SEN-GUPTA (**2**) studied the absorption spectra of chlorophyll components *a* and *b* prepared by STOLL, in acetone solution. They measured absorption coefficients with a König-Marten spectro-photometer. In so far as the spectrum is concerned their work was not very comprehensive, since they made only fifteen measurements from λ 4350 to 6900 Å.

Recently, WINTERSTEIN and STEIN (**14**) have reported the purification of both components *a* and *b* by the adsorption method alone. Their method and results for chlorophyll *a* are similar to those described in this paper, although their solvents and adsorptive agent were different. These workers also claim that chlorophyll *b* prepared by WILLSTÄTTER and STOLL'S method contains 15-20% *a*, and their method of removal of this impurity is that of repeated adsorption. WINTERSTEIN and STEIN studied the absorption spectra of chlorophylls *a* and *b* in ether solution by photographic and visual methods. They did not report the 4100 Å. band of component *a*, but found two bands at λ 6070 and 6230 Å., while the writer found only one band in this region, at λ 6125 Å. They report the 6630 Å. band of pure *a* in ether solution to be more intense than the 4320 Å. band. The reverse was found by the writer. The wave lengths of their absorption maxima differ from the values of table I by 5–215 Å. The smaller of these differences are undoubtedly due to errors of their methods of measurement. In the case of chlorophyll *b*, however, an absorption band is reported at λ 6140 Å. by WINTERSTEIN and STEIN (as well as by TSWETT). At this wave length the writer found a minimum of absorption for component *b* and a maximum for component *a* at λ 6125 Å. For this reason the *b* of WINTERSTEIN and STEIN probably contained a small amount of *a* as an impurity. They do not consider the possibility of a third "component *c*."

The methods of absorption spectra measurements employed by these workers were not sufficiently accurate and sensitive to find such small differences as can be detected with the photoelectric method. If the yellow pigments are completely removed by a technique such as WILLSTÄTTER'S, chlorophyll *a* may be isolated in a pure state by a modification of TSWETT'S adsorption method. Component *b*, however, has not been isolated completely from *a* by TSWETT'S method.

In the case of chlorophyll *a*, the pure blue color in ether solution

indicates its complete isolation from *b*. The absence of the 4525 Å. band is the chief reason for this blue color. Small amounts of *a* can-

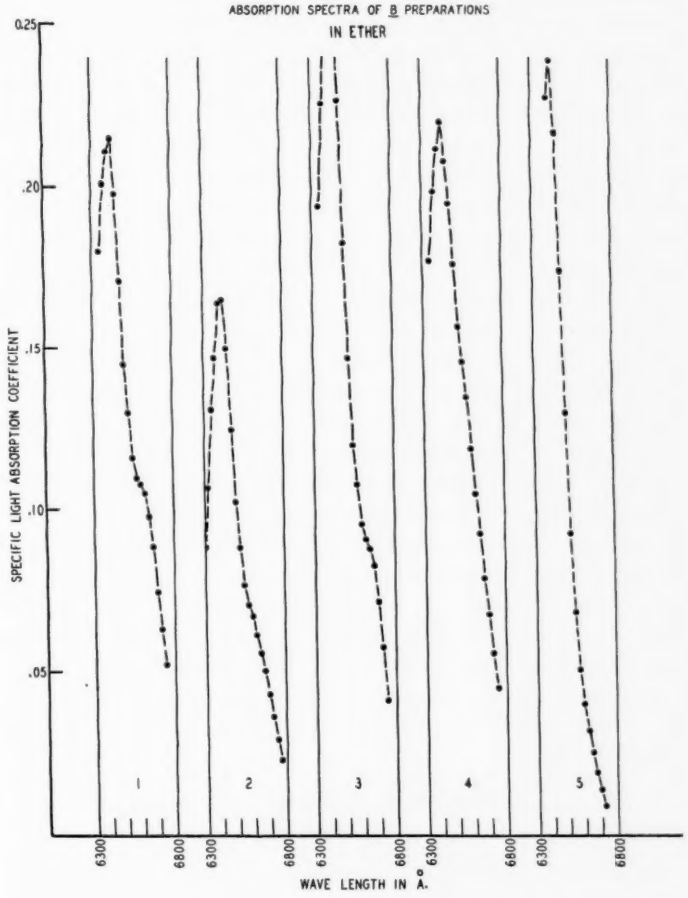

FIG. 2

not be detected in chlorophyll *b* preparations by color observations alone because their ether solutions are green. The best test of purity is an examination of certain small regions of the absorption spectrum

in the ranges of λ 4200–4800 Å. and λ 6300–6700 Å. for components *a* and *b* respectively. In these regions the absorption differences of the two components are greatest. Measurements should be taken at intervals of 25 Å. In figure 2 is a group of these test curves in which the specific light absorption coefficients are plotted as the ordinates. The first four curves (1–4) show the light absorption of *b* preparations from fresh nettle leaves (*Laportea canadensis*). The concentrations of these preparations in ether were only approximately known. Curve 1 is of extract group 1, consisting of extracts 1–4 (step VII 1). Curve 2 is of extract group 2, consisting of extracts 5–10 (step VII 1). In both of these preparations considerable *a* is present, as indicated by the change of slope in the absorption curves at approximately λ 6600 Å., where an absorption maximum of *a* occurs. The *b* of the third extract group, consisting of extracts 11–18, was purified by fractional precipitation (step VII 3, 4). Curve 3 presents the absorption of the fraction which is contaminated with *a* and penetrates the talc; curve 4, which presents the absorption of the precipitated fraction, does not have a change of slope at λ 6600 Å. At λ 6525 Å., a very slight change of slope indicates that probably a trace of *a* remained in this preparation. The amount of material available did not permit a further fractional precipitation of *b*. These curves (3 and 4) show that *b* is more easily precipitated than *a*, which tends to penetrate the talc.

Curve 5 is of *b* from extract group 3, consisting of extracts 11 to 18 (step VII 1). This preparation of *b* was made from fresh barley leaves and contained no *a*. In contrast to curves 1–3, there is no sharp break in the slope on the red side of the 6425 Å. band in the region of λ 6600 Å.

Quantitative analytical method for components *a* and *b*

A quantitative spectro-photoelectric method for analysis of mixtures of *a* and *b* has previously been described in detail (**15**). Values of α were accurately determined for pure *a* and *b* from the data presented in figure 3. From a measurement of $\log \frac{I_o}{I_x}$ at λ 4400 Å. (α has the same value for *a* and *b* at this wave length), the total chlorophyll (*a*+*b*) concentration is calculated. The ratio of *a* to *b* is then calcu-

lated from a measurement of α at λ 4100 or 4270 Å., by use of the equations of figure 3. In table II are the results of analyses of mixtures of *a* and *b*. From the experimental measurements of α for known mixtures, as presented in figure 3, the percentage composi-

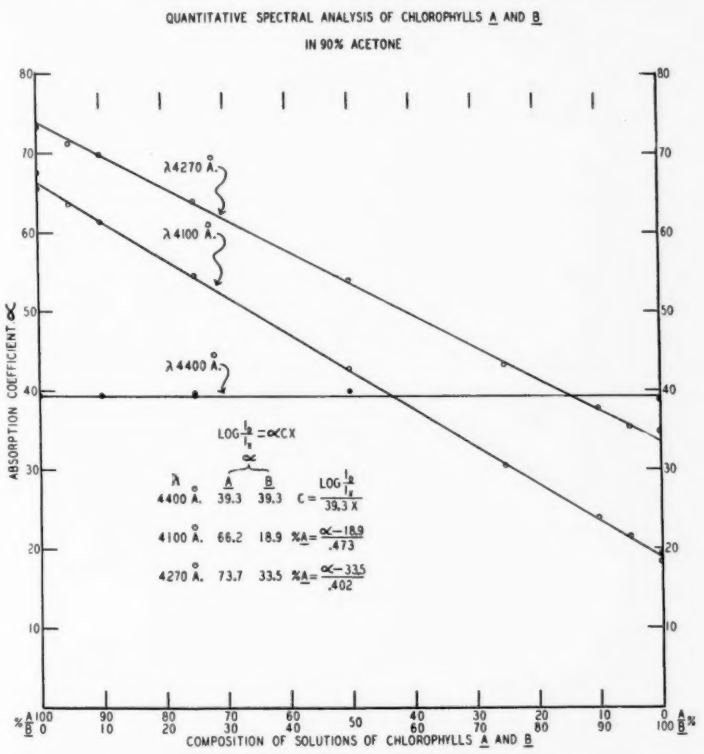
QUANTITATIVE SPECTRAL ANALYSIS OF CHLOROPHYLLS A AND B
IN 90% ACETONE
ABSORPTION COEFFICIENT ∝
λ4270 Å.
λ4100 Å.
λ4400 Å.
LOG $\frac{I_2}{I_1}$ = ∝CX
∝
λ A B
4400 Å. 39.3 39.3 C = $\frac{LOG \frac{I_2}{I_1}}{39.3\ X}$
4100 Å. 66.2 18.9 %A = $\frac{\alpha - 18.9}{.473}$
4270 Å. 73.7 33.5 %A = $\frac{\alpha - 33.5}{.402}$
%A 100 90 80 70 60 50 40 30 20 10 0
%B 0 10 20 30 40 50 60 70 80 90 100
COMPOSITION OF SOLUTIONS OF CHLOROPHYLLS A AND B

FIG. 3

tions of these mixtures were calculated. The values for the two wave lengths were averaged and the greatest deviation of this average from the true composition is 1.0%. Two unknown mixtures of *a* and *b*, in which neither the total chlorophyll (*a*+*b*) concentration nor the ratio of *a* to *b* was known, were analyzed by this method. The compositions, determined by measurements at λ 4100 and 4270 Å., agree

within 1.5%. It is therefore possible by this method to determine the percentage composition of unknown mixtures of components *a* and *b*, with an accuracy of 1.0% or better.

TABLE II

SPECTRAL ANALYSIS OF CHLOROPHYLLS A AND B IN 90% ACETONE

$$\text{LOG}\frac{I_0}{I_x} = \alpha C X$$

	α		COMPOSITION IN TERMS OF A									"C" FRACTION	
			KNOWNS A+B							UNKNOWNS A+B			
WAVE LENGTH	A	B	5%	10%	25%	50%	75%	90%	95%	1	2	10 MONTHS	24 MONTHS
4100 Å.	66.2	18.9	5.50	10.5	24.3	50.3	75.3	89.9	94.2	65.3	63.5	56.3	25.2
4270 Å.	73.7	33.5	4.73	10.4	24.1	50.7	75.6	90.3	93.8	66.7	65.0	40.1	23.6
AVERAGE % COMPOSITION			5.11	10.45	24.2	50.5	75.45	90.1	94.0	66.0	64.25	48.2	24.4

Solution color study

A study was made of the color of chlorophyll solutions having approximately the same color intensity (table III).

The differences between the colors of *a* and *b* are most pronounced in ether, less in ethyl alcohol, and least in carbon bisulphide. The colors of fraction c are intermediate between those of *a* and *b*. Ether and ethyl alcohol solutions of chlorophyll have a brilliant red fluorescence. Carbon bisulphide solutions do not fluoresce visibly. WILLSTÄTTER usually refers to the color of chlorophyll *a* solutions as blue-green or greenish blue, and only once (**13**, p. 151) does he state that "the concentrated ethereal solution may be called pure blue."

TABLE III

SOLVENT	*a*	*b*	FRACTION C
Ether (anhydrous)	*Blue*	Yellowish green	Green
Ethyl alcohol (absolute)	Slightly greenish blue	Yellowish green	More yellowish than in ether
Carbon bisulphide	Green, almost like fraction c in ether	Almost yellow	Yellowish green like *b* in ethyl alcohol

Pheophytins of *a*, *b*, and fraction c were made by the addition of concentrated ether solutions of chlorophyll to 95% ethyl alcohol, to which were then added a few cc. of 22% hydrochloric acid. After one

hour, the alcohol was extracted with water and the ether solution stood overnight with 22% hydrochloric acid. The pheophytins were transferred to ether by dilution and washed with water. Dilute solutions of the pheophytins were compared (table IV) as to their color, phase, and later phase (color a few minutes after addition of methyl alcoholic potassium hydroxide).

TABLE IV

Solvent	*a*	*b*	Fraction c
Ether solution	Bluish brown	Yellowish brown	Yellowish brown
Phase	Yellow	Pink	Pinkish brown
Later phase	Greenish	Brown	Brown

The properties of the pheophytin of fraction c are somewhat intermediate between those of *a* and *b*. The later phase colors of *a* and fraction c are particularly different. Ether solutions of the free isochlorophyllides of *a* and fraction c are distinctly different in color: that of *a* is violet-blue and that of fraction c is pure green. Aqueous solutions of *a* and fraction c, previously saponified with hot methyl alcoholic potassium hydroxide, are blue and green respectively, and fluoresce brilliantly. When barium chloride is added to such aqueous solutions, the barium salts are precipitated. These barium salts of *a* and fraction c are purple and green respectively.

Tests of purity

1. Basicity test.—The phytol ester group was undamaged in all of these preparations, because 22% hydrochloric acid extracted no color from their ether solutions.

2. Phase test.—The writer has found that the phase test is useless for detection of contamination of *a* in *b*, or vice versa. The color of the phase is subjective and depends to a great extent upon the concentration of chlorophyll. The phase of *a* is yellow and that of *b* is pink, if the proper concentration is used. The phase of fraction c is usually yellow, but that of some preparations is reddish yellow or brown.

3. Presence of yellow pigments test.—In the phase test, after complete saponification of the chlorophyll (several hours) in ether solution with methyl alcoholic potassium hydroxide, ten volumes of water are added; the saponified chlorophyll remains in the aqueous layer and the yellow pigments color the ether layer, if they are present. When this test

is applied to the components prepared by the preceding method, the ether layers are colorless.

4. CLEAVAGE TEST.—These preparations did not behave in the cleavage test in the same manner as did WILLSTÄTTER'S preparations, according to his descriptions. It required 22% hydrochloric acid to extract all of the color from the ether solutions of the phytochlorins from *a* and fraction c and the phytorhodin from *b*. Only a part of the color was extracted by 4%, 9%, and 12% hydrochloric acid.

Since all of the chlorophyll preparations gave good phase tests, it seems that the chlorophyll was not allomerized and that allomerization was not the cause of the formation of weakly basic products in the cleavage tests. Possibly the preparations were not saponified under the proper conditions.

5. ABSORPTION SPECTRUM TEST.—A critical examination of the absorption spectrum, measured with the accuracy of those in figure 1, is the best test for contamination of *a* or *b* by each other or another component. This method of measurement is not subjective, and is very accurate and precise.

SOURCE OF LEAF MATERIAL

Fresh leaves were the source of chlorophyll. They were ground and extracted as quickly as possible after harvest, often within 15 minutes. For most of the preparations barley has been used, because it is conveniently and rapidly grown and has proved very satisfactory. It contains less of the non-chlorophyll pigments than do the other plants tried, and the preparation is therefore easier. Wisconsin barley, Pedigree 38, age 1 month, height 10 inches, was used.

Components *a* and *b* have both been isolated from barley and from nettle (*Laportea canadensis*) in the pure state. The blue component, *a*, has also been isolated from the leaves of Kentucky bluegrass (*Poa pratensis*) and Australian rye grass (*Lolium perenne*) from the lawns of the University of California campus, from foxtail (*Hordeum jubatum*), thimbleberry (*Rubus*), and spinach. The typical blue and green zones of step VI 4 were formed from chlorophyll derived from each of these sources (table V). In the vacant spaces of the table, the yields were not measured.

The yields from grass are the average of three preparations. No *a* and fraction c were recovered from the methyl alcohol extracts. The total of *a* plus fraction c is 0.8 gm. per kg. of fresh grass. Both *a* and fraction c would have been included under component *a* by WILLSTÄTTER and their total yield is the same as his yield of *a* given in his example (**13**, p. 127),

in which he obtained 4.05 gm. of chlorophyll ($a+b$) from 2.5 kg. of fresh nettle leaves. From 8.0 gm. of chlorophyll he obtained 3.7–4.0 gm. of component a, or about 0.8 gm. of a per kg. of fresh leaves (**13**, p. 150).

TABLE V

YIELDS OF PURE CHLOROPHYLL COMPONENTS; APPROXIMATE YIELDS (IN GM.) PER KG. OF FRESH LEAVES

SOURCE	*a*	*b*	FRACTION c
Barley	0.1300	0.0145	.....
Barley (more succulent)	.....	0.0070	.....
Lawn grass	0.4200	.....	0.3800

TIME OF PREPARATION

Harvesting and grinding of the leaves, acetone extraction, transference of the chlorophyll to petroleum ether, and removal of excess acetone require about 1.25 hours. The methyl alcohol extractions of b require 1.25 hours if these extracts are discarded and a is the only component purified; 2 additional hours are necessary if these extractions are to be worked up for b. The mixture of a and fraction c can be precipitated within 2 hours and their separate ether solutions obtained within 2 additional hours. The time elapsed from the harvest of the fresh leaves to the pure ether solution of a is thus about 6.50 to 7 hours. More time is required to isolate pure b.

Comparison with other methods of component purification

Since the present method is a combination and elaboration of the methods of WILLSTÄTTER and STOLL and of TSWETT, the following discussion will indicate points of difference between the methods, and will present reasons why the contamination of a with b (or component c) was not suspected by previous workers.

According to WILLSTÄTTER and STOLL, step VI 2 results in a solution of pure component a. That this preparation may be further separated into *blue* and green portions is evident from the talc adsorption experiments. In their method, after the a is quantitatively precipitated in petroleum ether by washing out the methyl alcohol, talc is added to collect the chlorophyll particles for filtration. If a small part of the "chlorophyll a" were not precipitated, but remained in solution, this added talc would adsorb it and thus prevent

its later separation into zones during filtration. Also, *a* is more easily precipitated from petroleum ether than is fraction c, the green portion, so that the more completely the mixture was precipitated the thinner would be the blue zone of *a*, and the color difference would be less easily noticed. In their method for preparation of pure chlorophyll (*a*+*b*), the precipitated pigment "is immediately dissolved from the talc upon the suction filter" with ether (**13**, p. 123). Thus no opportunity is given for observation of zone formation by any chlorophyll that might not have been precipitated and collected in the top black layer. In their separation of *a* and *b*, the final precipitate of *a* on talc is dried as well as possible with suction. As the talc becomes drier the difference in color of the *a* and c zones becomes less obvious, and both layers become gray. From these considerations it is clear that the more complete is the precipitation of chlorophyll *a* and the more carefully the method of WILLSTÄTTER and STOLL is followed in several details, the less chance has the experimenter of observing the separation of the blue and green fractions. If TSWETT had used an ether-petroleum ether mixture instead of carbon bisulphide as a solvent, more efficient separation of the blue from the green layers could have been made, because the color differences of *a*, *b*, and fraction c are much less in carbon bisulphide solution.

The purification of *b* is very wasteful, but results in a pure product. In the method of WILLSTÄTTER and STOLL, *b* is precipitated from 10 cc. of ether with 400–500 cc. of petroleum ether; while the ratio in the writer's method is 13 cc. of ether to 100 cc. of petroleum ether. All of the methyl alcohol washings (VII) are not used, thus the yield of *b* is decreased further. A less wasteful method for the isolation of *b* in a pure state is much needed for further studies in which larger amounts of *b* will be required.

Number of components and sources of error

It is the opinion of the writer that there is at least one component in the chlorophyll complex, other than components *a* and *b*. Chlorophylls *a* and *b* have been more highly purified than before. This third component, *c*, has not been isolated in a pure state from fraction c, which contains component *a* as an impurity. Consideration of the appearance of two chlorophyll fractions, distinctly different in

color and separated from the preparation formerly considered as chlorophyll *a*, immediately suggests a number of questions regarding possible sources of error in the preparative methods:

(1) Have *a* or component *c* been formed as a result of chemical change of WILLSTÄTTER'S *a* during the purification procedure?

(2) Since the color of fraction c is intermediate between those of *a* and *b* in ether solution, is fraction c a mixture of *a* and *b*, and is its appearance due to incomplete extraction of *b* from *a*?

(3) Is component *c* a polymer or associated product of *a* or vice versa?

(4) Do the fractions *a* and c have different colors because of the presence of some other blue or yellow pigment respectively?

To answer these questions, the components were studied from several points of view, both chemical and physical, and the preparative methods were varied to exclude error. These questions will be considered in turn.

(1) The use of fresh leaves avoids decomposition of chlorophyll due to heating or drying processes. None of the solutions or extracts were heated above room temperature at any time. In the plants studied, acids of the sap do not decompose the pigment during grinding and extraction from the leaf meal. This is evident from the fact that pheophytin does not appear in the final products. Pheophytin would be detected very easily by its strong absorption bands at λ 5000 and 5300 Å. for *a* and *b* respectively, which are located where the pure components absorb least. The pure blue and green colors of the component solutions would thus be changed considerably by the brown pheophytin if it were present. Because of the possibility that a rearrangement of *a* might be catalyzed by the tin walls of the press or the enameled wall of the pan during the extraction, the press and pan were not used in certain preparations. The extract was pressed entirely by hand from the meal into Pyrex beakers. These variations did not alter the results. With some extractions no oxalic acid was used in the 85% methyl alcohol and good preparations resulted, but in other such cases allomerization occurred; therefore oxalic acid was used in all further preparations.

Fresh solvents were used throughout many preparations. Properly reclaimed solvents give equally good results. To make certain that no impurity in any of the solvents was responsible, through oxidation or decomposition, for the appearance of the *a* and c zones in talc, a preparation

was made in which only specially purified solvents were used. Following is a description of the purification methods employed and of the tests applied to the solvents.

Methods of purification of solvents

Acetone (c.p.) was distilled over aqueous Na_2CO_3 and Na_2SO_3 solution, through an 18-inch fractionating column b.p. 56.5° C., odor sweet.

Petroleum ether (Oronite), after standing over aqueous $KMnO_4$ overnight, was distilled. sp. gr. 0.656, b.p. 36–75° C., 50% distilled below 50° and 75% below 60° C. Another grade of petroleum ether, with the following distillation temperatures, was very satisfactory: 50% at 33–40° C., 45% at 40–50° C., and 5% at 50–60° C. The low-boiling petroleum ether distilled as follows: 50% at 31–32° C., 40% at 32–33° C., and 10% at 33–34° C.

Methyl alcohol (12 liters) was treated with 60 gm. of zinc dust and 80 cc. of 50% NaOH solution, boiled under a reflux condenser for 0.75 hour, and then distilled through a 5 ft. fractionating column.

Ether (Baker's anhydrous) stood over sodium chips overnight. It was then distilled over fresh sodium and used immediately.

Tests applied to solvents

The $KMnO_4$ and KI tests were made in closed containers in the dark.

Acetone. (a) For aldehydes: Heat 10 cc. of acetone plus 5 cc. of ammoniacal silver nitrate solution (protected from light) for 15 minutes in a water bath at 50° C. If aldehydes are absent, the mixture should not acquire a brown color and no metallic silver should appear.

(b) Add 1 drop of 1/1000 $KMnO_4$ solution to 10 cc. of acetone and keep below 15° C. The pink color should not disappear entirely in 15 minutes.

Methyl alcohol. (a) For aldehydes: Add 1 drop of N/10 $KMnO_4$ to 10 cc. of methyl alcohol. The red color should not disappear within 10 minutes.

Ether. (a) For ethyl peroxide, hydrogen peroxide, or ozone: Shake 10 cc. of ether with 1 cc. of N/10 KI solution. No color should be acquired after standing 1 hour in the dark.

Other tests used on all solvents were the following:

I. For oxidizing agents: A few drops of neutral KI solution are shaken with 10 cc. of the solvent and allowed to stand for 1 hour. A coloration indicates the presence of an oxidizing agent which oxidizes I^- in neutral solution.

Ia. To I, a few drops of dilute H_2SO_4 are added, shaken, and allowed to stand. The H^+ greatly sensitizes I.

II. For peroxide: A few drops of $TiCl_4$ solution added to 10 cc. of the solvent tested produces a yellow or red coloration if peroxides are present.

III. For aldehydes: Add a few drops of Shiff's reagent to 10 cc. of the solvent tested. A red coloration indicates the presence of aldehyde.

The following tests on the solvents were negative:

ACETONE.—a, b, I, II, and III. Ia was negative for 0.50 hour (slight coloration overnight).

PETROLEUM ETHER.—I, II, and III. Ia was negative for 0.50 hour (slight coloration overnight).

METHYL ALCOHOL.—a, I, II, and III. Ia gave a slight coloration in 0.50 hour and much color developed overnight.

ETHER.—a, I, II, and III.

Excellent preparations of *a* and fraction c were made with solvents purified by the methods just described. Some *a* was dissolved in acetone and left 2 days in the dark. After transference to ether it still gave a good yellow phase test and retained its original blue color.

(1) and (2). Ether solutions (0.1 gm./liter) of *a* are *blue* and cannot be called greenish blue or bluish green. Ether solutions of *b* are yellowish green and those of fraction c are green. The apparent colors of green solutions are somewhat subjective and depend considerably upon the comparison solution. Solutions of fraction c might be called bluish green in comparison with those of *b* if certain concentrations are used. Mixtures of *a* and fraction c are definitely bluish green when compared with *b*. If solutions of *a* and *b* having approximately equal color intensities are used to make one of the same shade of green as fraction c, the mixture must be about two-thirds *a* and one-third *b*. That fraction c was a mixture of *a* and *b* after twenty extractions with 85% methyl alcohol seemed very improbable; nevertheless several experiments were performed to prove that this was not the case and that *a* and fraction c were not changed into one another during the alcoholic extractions and subsequent filtrations.

Fraction c from two preparations was dissolved in ether, added to 1 liter of petroleum ether (b.p. 35–55° C.), and extracted twenty times with 85% methyl alcohol and once with 90% methyl alcohol. The pigment remaining in the petroleum ether was filtered on talc (step VI 4) and gave a thick green layer of fraction c and a very thin blue layer of *a* below, amounting to about 5% of the total. This *a* was evidently not separated from component *c* in the first filtration, which was shown by the fact that

when fraction c, prepared by the usual twenty extractions with 85% methyl alcohol, was dissolved in an ether-petroleum ether mixture and filtered, a very thin bluish layer was found at the bottom of the colored talc. When *a* was extracted twenty additional times with 85% methyl alcohol and filtered on talc in the same manner as in the usual preparation, no green layer was found and the blue zone occupied the total colored portion of the talc. The products left after these last extractions had been extracted forty times with 85% methyl alcohol and three times with 90% methyl alcohol. These may be compared to WILLSTÄTTER and STOLL'S sixteen extractions with 85% methyl alcohol and three with 90% methyl alcohol, which supposedly completely removed *b*.

WILLSTÄTTER and STOLL (**13**) did not state whether the compositions of their 85% and 90% methyl alcohol solutions, used for separation of *b* from *a*, were expressed in volume or weight percentages. It has been assumed that volume percentage was used. A preparation was made, however, in which the methyl alcohol solutions were prepared on the weight percentage basis. The methyl alcohol extracts were all darker in color than when the solutions prepared on the volume percentage basis were used. Only sixteen extracts with 85 weight % methyl alcohol were made, as directed by WILLSTÄTTER and STOLL'S method. The sixteenth extract was rather dark. The petroleum ether solution was much more dilute than usual after the sixteenth extract. Three extractions with 90 weight % methyl alcohol, which were dark colored, left the petroleum ether solution definitely bluish in color but it was not the pure blue characteristic of pure component *a*. In step VI 4, the layers of *a* and fraction c were not sharply defined in the talc because the ether concentration was too great. The upper portion was greener than the lower portion, however, and the two ether solutions were different in color, that of the top layer being greener than that of the lower. The yield of *a* plus fraction c was less than usual, and the yields of the first crude *b* fractions were correspondingly greater, because they contained more *a*, which appeared in step VII 3. This experiment demonstrates that even when the 85% and 90% methyl alcohol solutions are prepared on the weight basis, the green c fraction is not completely separated from the *a* and the yields are low.

(3) After saponification in the phase test, when water is added to the mixture, the ether becomes colorless and the lower aqueous solution is blue in the case of *a* and green in the case of fraction c. Alkalies often cause polymerization in solution. Since the aqueous layers, formed by additions of water to the phase tests, remain blue with *a* and green with

fraction c, retaining their color differences, and since *a* and fraction c pheophytin solutions, which have been treated with acid, have different colors, it is very improbable that component *c* is a polymer of *a* or vice versa.

(4) Since the ether solution becomes colorless after addition of water to the phase test of fraction c, the green color is not caused by the presence of carotene or xanthophyll as an impurity of *a*. Xanthophyll esters would be hydrolyzed by the alkali, liberating the xanthophyll to the ether layer. The blue color of *a* cannot be due to an anthocyanin impurity, because the anthocyanins are insoluble in ether, benzene, and other solvents in which chlorophyll is soluble (**12**, p. 46). The green color of fraction c is not due to an anthoxanthin impurity, since the anthoxanthins and flavones dissolve in water and acids to give yellow and red solutions.

Discussion of fraction c

Efforts were made to prepare the green component *c* of fraction c in a pure state by repeated filtration of the ether-petroleum ether solution of fraction c through talc and separation of the blue *a*. The writer was unable by this means to isolate from fraction c a green component whose properties were constant. The green product always exhibited properties of a mixture.

Fraction c was prepared from fresh foxtail (*Hordeum jubatum*) leaves. In step VII the petroleum ether solution was washed forty times with 85% methyl alcohol, and in step VII 3–6 three filtrations through talc were made to separate the last traces of *b* and *a* respectively. The third talc chromatogram had a very thin blue layer of *a*, about 5% as thick as the green layer. The absorption spectrum of an ether solution of this fraction c, after three adsorption purifications, was examined visually with a small spectroscope. It contained two distinct bands of about equal intensity in the red region, such as would be shown by a suitable mixture of *a* and *b*. The same absorption spectrum was obtained from the fraction c of a preparation from lawn grass after three filtrations through talc (step VI 3–6). When the fraction c from the foxtail was one year old, having been carefully dried and kept in a weighing bottle in darkness, its absorption spectrum was measured in a more precise manner with the spectrophotoelectric method described earlier (**16**). In general shape the absorption curve was intermediate between those of *a* and *b*. The

bands at λ 4525 and 6425 Å. were predominant over those at λ 4275 and 6600 Å. This indicates that *if* fraction c were a mixture of *a* and *b*, *b* was present to the extent of about 75%. Since (1) the last ten of the 85% methyl alcohol washings were made after the second talc filtration and were very light in color, and (2) only about 5% of the total was removed as *a* in the third filtration, it is very improbable that 75% *b* could have been present in the final preparation. This is evidence that fraction c is not simply a mixture of *a* and *b*, but contains a third component *c*.

These preparative data indicate that at least one component other than *a* and *b* exists in the chlorophyll complex. Although it has not been prepared in a pure state, certain of its properties may be deduced from the foregoing data. Its color must resemble that of *b*. With respect to the separation between petroleum ether and methyl alcohol, this third component *c* has properties similar to those of chlorophyll *a;* considering differential adsorption on talc, its properties are similar to those of *b*.

Melting point data are not reliable as criteria of purity for the chlorophyll components. The sintering and melting points vary considerably with the speed of heating and are often very indefinite.

Two preparations of fraction c were analyzed as unknowns. The results are presented in the last two columns of table II. These samples were not fresh but had been dried, powdered, and kept in a weighing bottle in the dark for periods of 10 and 24 months respectively. Both samples were prepared according to the method here presented, the 10-month sample being purified twice by steps VI 3–6 and the 24-month sample three times by steps VI 3–6 plus ten additional washings with 85% methyl alcohol in step VII. The 10-month sample was isolated from barley leaves and the 24-month sample from the leaves of a closely related plant, foxtail.

The analyses were made in the same manner as were those of the unknowns containing *a* and *b*, on the assumption that fraction c contained a mixture of *only a* and *b*. The total concentration was determined by a measurement of $\log \frac{I_0}{I_x}$ at λ 4400 Å. From the determinations of a at λ 4100 and 4270 Å., for *a* and *b*, the percentage of *a* was calculated by use of the equations of figure 3. If only *a* and

b were present, the results from measurements at the two wave lengths should agree within 1.5%. In the case of the 10-month sample, however, the calculated compositions disagree by 16.2%, which is many times the experimental error. This disagreement proves that there is a third substance which does not absorb light to the same extent as either *a* or *b* at one or more of these wave lengths. It is the opinion of the writer that this third substance is a third component, *c*, of chlorophyll. However, there may be more than one component other than *a* and *b*. These data show only that there are more than the two components *a* and *b* in the chlorophyll complex and set no upper limit to the total number. In the case of the 24-month sample the agreement is fairly good, within 1.6%, as though only *a* and *b* were present. This agreement indicates that component *c* may change to *b* on standing. A sample (*a*+fraction c), as obtained in step VI 2–3, was kept in a dry powdered condition for 3 years. It originally gave a chromatogram of two zones, 1 and 2 (pl. X, B). After 3 years its chromatogram consisted of three distinctly different zones, similar to zones 2, 3, and 4 of plate X, A. The upper one was dark green, the middle zone lighter green, and the lower zone greenish blue. Evidently the *a* had somewhat deteriorated, as its color was not pure blue. The respective zone thicknesses were in the ratio 5:12:17. This is further evidence that component *c* may change to *b* on standing. The nature of this change and its optimum conditions are unknown.

Summary

1. An improved method for the isolation of chlorophyll components *a* and *b* has been developed. The methods of WILLSTÄTTER and STOLL and of TSWETT were combined and modified. The final purification processes involve differential adsorption on talc and fractional precipitation from petroleum ether, for components *a* and *b* respectively. The steps of the procedure are presented in detail.

2. The absorption spectra of chlorophylls *a* and *b* were measured accurately from λ 3950–7800 Å. by a photoelectric method. Absorption coefficients at the maxima and minima were determined with an accuracy of 1.0%. The visible spectrum of each component consists of seven bands.

3. Spectral data indicate that the components prepared by WILL-

STÄTTER and STOLL and by TSWETT were not separated completely from one another. The spectral method for detection of impurities is discussed.

4. A comparative study was made of the colors of *a* and *b* in various solvents and of certain chlorophyll derivatives.

5. Different tests of purity are discussed and it is concluded that a critical examination of the absorption spectrum is the most reliable and sensitive test.

6. Sources of leaf material and component yields are presented.

7. The improved preparation procedure is critically compared with previous methods.

8. It is suggested that there is at least one component other than chlorophylls *a* and *b* in the chlorophyll complex. Possible sources of error in the purification procedure are discussed.

9. Attempts to isolate the third "component *c*" were unsuccessful. Its properties are intermediate between those of components *a* and *b*.

10. Quantitative analytical data affirm the existence of a third component *c*.

This research was started at the Laboratory of Plant Nutrition, University of California, in 1929 and continued at the George Herbert Jones Chemical Laboratory, University of Chicago, from 1931 to 1933. The absorption spectra and methods of purification were presented before the Division of Biological Chemistry at the 85th Meeting of the American Chemical Society, Washington, D.C., March, 1933.

The writer wishes to express his appreciation to those who have given him the opportunity for this investigation: in particular to Professor D. R. HOAGLAND, of the University of California, under whose direction the first work in the purification of chlorophyll *a* and the attempted isolation of component *c* were made; to Professor T. D. STEWART for suggestions on solvent purification; to Professor T. R. HOGNESS, of the University of Chicago, for many suggestions of technique in the absorption spectra measurements; to Dr. E. S. MILLER for making a preparation of component *a* from directions written by the writer to demonstrate that another experiment-

er, unaccustomed to the technique, could secure the same results; and lastly, to the Board on National Research Fellowships in the Biological Sciences for making the latter part of this work possible by the grant of a fellowship.

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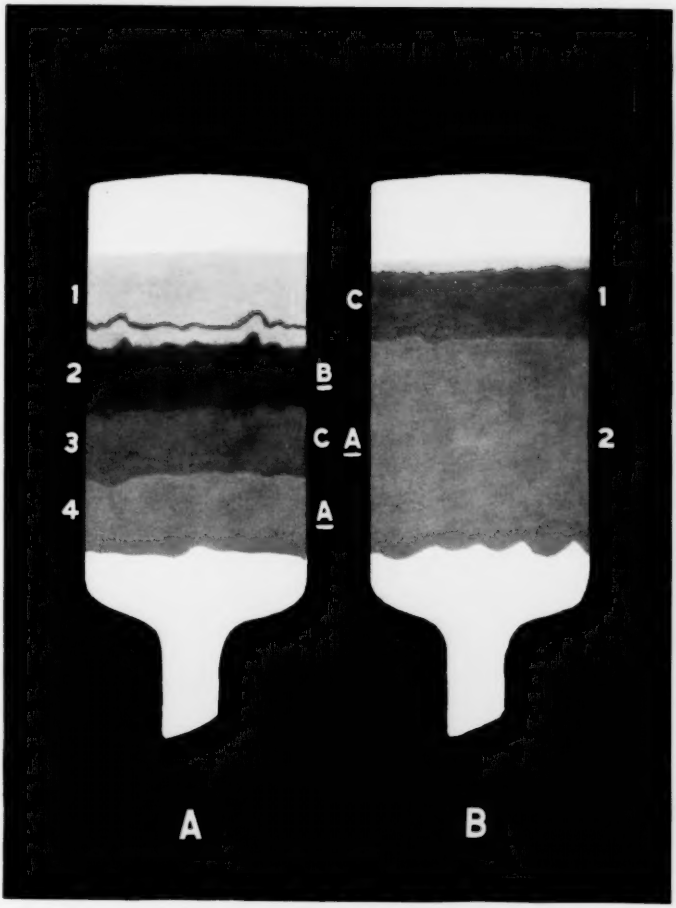


ZSCHEILE on CHLOROPHYLL PURIFICATION

DEVELOPMENT OF SPORE WALLS IN SPHAEROCARPOS DONNELLII

MARGARET B. SILER

(WITH TWENTY-SIX FIGURES)

Introduction

The persistent tetrads of spores derived from each spore mother cell, which are characteristic of most species and races of *Sphaerocarpos*, were interpreted by BISCHOFF (**6**) in *S. terrestris* as three-knobbed spores. PETOUNNIKOW (**23**), realizing that each tetrad is composed of four spores, called these tetraspores because of their resemblance to the tetraspores of the Florideae. LEITGEB (**18**) and later writers have referred to them as spores persistent in tetrads.

AUSTIN (**3**) first described *Sphaerocarpos donnellii* from Jacksonville, Florida. According to his description the species has a fragile, deeply lobed coccus [tetrad], each spore of which possesses a large, fragile tubercle. HAYNES (**15**) and HAYNES and HOWE (**16**) cite among the specific characters of *S. donnellii*, "spores separating at maturity, ... each while united in the tetrad commonly showing a prominent protuberance about 12 μ high in middle of outer face and after separation exhibiting a coarsely lobed basilar margin." A species whose spores separate early in their development, *S. cristatus*, has been described from California (**8, 9, 17**).

ALLEN (**2**) finds that in most of his cultures of *S. donnellii* the spores remain united in tetrads. In a few cultures, however, the spores separate at maturity, somewhat as described for the species by the taxonomic writers. Studies of matings between plants producing sporophytes whose spores remain united at maturity and those in whose sporocarps they separate show that among the descendants of one clone (R27E) the separate-spore tendency is regularly transmitted through the female gametophyte (**2**). This character follows in inheritance the course of the X-chromosome (**1**).

Later examination of herbarium material from the type locality for the species (Jacksonville) disclosed the presence of some sporo-

phytes with separate or separable spores and others with persistent tetrads. Plants of *S. donnellii* received from Tallahassee, Florida, in the spring of 1929 had sporophytes with separable or rarely adherent spores; plants from Gainesville, Florida, received the same spring had sporophytes with adherent spore tetrads. Cultures have been made from these two lots of material.

The accomplishment of normal matings (**2**) between gametophytes from separable and those from non-separable strains suggests that the adherence or non-adherence of spores produced by sporophytes from the respective types of female is due to the presence of one or the other of a pair of allelomorphic genes, which, at least in culture, affect spore wall development. One object of the present study was to determine what, if any, feature in the history of development of the spore wall is responsible for the separation or adherence of the spores.

As early as 1867, PETOUNNIKOW (**23**) inquired into the structure and development of the "tetraspores" of *S. terrestris*. According to him the spores remain united in fours, surrounded by the walls of the "special mother cells," which become transformed into a reticulate, cuticular envelope. When the septa between spores are apparent, sections of the tetrad wall show three layers: the outermost, yellow at maturity with ridges forming a reticulum, and extending between the spores by means of the septa; the innermost, lining each spore; and a third lying between these two layers. The septa formed by the first layer between the spores do not show a distinct separation into layers belonging respectively to each of the adjoining spores.

LEITGEB (**19, 20**) agreed with PETOUNNIKOW in interpreting the wall inclosing the tetrad of *S. terrestris* as the transformed "spore mother cell (walls of the special mother cell)." The common outer wall is divided by a network of ridges, caused by folds in the wall membranes, which have "acute spicules" (**15**) at the intersections. Where this outer wall continues between the spores it is granular and without a middle lamella. Within the outer wall each spore of the tetrad is surrounded by its own exine and cellulose intine. The exine of each spore has a reticulate pattern corresponding to that of the outer wall. The intine has no special structural details. LEIT-

GEB'S description of the formation of these walls will be discussed later. His paper of 1883 (**19**) was a preliminary account without illustrations. The more extensive and illustrated account of the spore walls of *Sphaerocarpos* (**20**) to which STRASBURGER refers repeatedly has been unavailable.

STRASBURGER (**26**) concluded that the spores of *S. terrestris* remain united in tetrads because each tetrad is surrounded by a continuous outer layer of exine. At maturity each spore, inside the common outer exine, is surrounded by a dark brown, finely lamellate layer (the inner exine) and a thick homogeneous intine. The inner exine projects in the ridges of the spore pattern on the outer faces of the spores, but is thin in the septa between spores, being somewhat thicker between the outer margins and between the apices. STRASBURGER distinguished a middle lamella in the septum. The development of the persistent tetrad will later be compared with that found to occur in *S. donnellii*. The correspondence of the outer exine of the tetrads of *S. terrestris* to the outer exine of *Riccia glauca* was pointed out, with the conclusion that this layer is an exine, not a perinium.

LORBEER (**21**) notes a few features in the development of the tetrads of *S. donnellii*, employing LEITGEB'S terminology (**20**). Exine and intine originate about each spore. The whole tetrad complex in the meantime becomes surrounded by an increasingly thick gelatinous layer which is apparently built up with the help of the original spore mother cell wall and the nurse cells. In the midst of this layer the undulate structures of the perinium become apparent. The perinium holds the spores fast together.

Riccia is the only other genus of hepatics in which the spore wall development has been studied in any considerable detail (**20, 26, 4, 7, 10**). STRASBURGER (**26**) found three layers in the mature spore wall of *Riccia glauca:* an outer and an inner exine, whose folds are coincident except where they are separated by granular material about the basal margin of the spore, and an intine. The division of the spore mother cell is followed by a thickening of the special mother cell wall (that is, the walls surrounding and separating the spores). The thickening proceeds in pillow-shaped areas with depressions between, into which the cytoplasm extends. Upon these thickening

layers each spore deposits a wall of its own. This layer thickens and then a second layer is laid down by the cytoplasm. As the inner exine thickens it becomes yellow, both it and the outer exine becoming cutinized and brown at maturity. Shortly before ripening of the spores the cytoplasm lays down an intine. The thickening layers of the special mother cell wall are finally entirely resorbed. Each spore during its development doubles its diameter.

STRASBURGER did not agree with LEITGEB'S (**20**) interpretation of the outer exine as a perinium derived from the special mother cell wall by transformation. He held that the earlier development of the outer exine as a tetrad-inclosing layer about the spore mother cell in *Sphaerocarpos terrestris* accounts for the persistence of tetrads in this plant, as contrasted with the separation of the spores of *Riccia glauca*, where the outer exine is laid down around each spore.

BEER (**4**) re-examined the development of the spore walls of *R. glauca*. In general he confirmed STRASBURGER'S conclusions. He did not commit himself regarding the origin of the first spore wall (outer exine), however, but held the evidence insufficient to show whether this layer is deposited upon the thickened special wall or is developed by transformation from the inner part of the thick special mother cell wall. The second spore wall (inner exine) becomes differentiated into three regions (two being lamellated with a structureless dark-staining layer between). An endospore (intine) is laid down late in the development of the spore. BEER concluded from chemical tests that the thickened special mother cell wall is composed of callose, that the first and second spore walls become cuticularized, and that the endospore is of pectose and cellulose.

BLACK (**7**) compared the development of spore walls of *Riccia frostii* with that of *R. glauca* as described by BEER. A few points concerning spore wall development are given by CAMPBELL (**10**) for *R. trichocarpa* and *Fimbriaria californica*, and by HAUPT (**14**) for *Reboulia hemisphaerica*.

Materials and methods

For this study of the development of spore walls in *Sphaerocarpos donnellii* Aust., matings of various clones were made; the sporophytes were collected and killed in Flemming's medium solution

upon successive days throughout the period of spore development. Sections were cut 7 μ in thickness and stained with Flemming's triple stain, safranin and light green, Haidenhain's haematoxylin and light green, or with methylene blue. The triple stain and the methylene blue gave the best differentiation of wall layers.

The plants used in matings were from ALLEN's clones and included the following:

♀	♂
31.453 (typ.[1])	×31.745
23.107 (typ.)	×31.773
30.613 (sep.)	×31.1021
29E3 (sep.)	×31.433
25.2409 (sep.)	×31.710
22.22 (sep.)	×31.745
20.22 (sep.)	×31.1021

The typical female 23.107 was one of the offspring of female 21.215 in a mating the sporophytes from which produced spores adhering in tetrads (**2**); 29E3 and 30.613 were females grown from separate spores from plants from Tallahassee. Females 25.2409, 22.22, 20.22 were descendants of the female clone R27E which always produced sporophytes with separable spores (**2**). All the males used were descended more or less remotely from a common female ancestor R35G, whose sporophytes always produced tetrads. One male ancestor, 20.45, of all the males was grown from a separate spore produced by a sporophyte derived from the female clone R27E.

Female and male plants to be mated were placed in the same pot on December 13, 1931, and flooded with sterilized water December 27. The fixations which supplied the material for study of the spore walls were made almost daily from January 26 to February 7, and on February 17, 1932, the sporophytes being 30–42 days and 52 days old respectively.

All drawings were made with the aid of a Spencer camera lucida. Figures 1 and 2 were drawn with a 12× planoscopic ocular and a Spencer 4 mm. objective. All other figures were drawn with a 12× planoscopic ocular and a Spencer 1.5 mm. objective.

[1]Typ., spores in tetrads; sep., spores separating.

Observations

Spore mother cells

The fully developed sporophyte of *Sphaerocarpos donnellii* resembles closely that of *S. terrestris* (*S. michelii*) which is often figured (**13**, fig. 214; **22**, fig. 51). The simplicity of the capsule with its single-layered wall (fig. 20), the narrow, short seta, and the bulbous absorbing foot have been abundantly commented upon (**10**, **11**, **22**, **24**).

In the sporophytes here under consideration the central sporogenous tissue of the capsule was separated into rounded cells as early as 19 days after fertilization. These free cells are suspended in a liquid medium in which they continue to develop and grow as the capsule enlarges. During this period of growth a differentiation takes place. Some of the free cells remain comparatively small, green, and packed with starch; others continue to enlarge and gradually lose their chlorophyll and most of their starch content. The former are nutritive cells,[2] the latter spore mother cells.

In either typical or separable races the cytoplasm of a spore mother cell having a diameter of 45 μ is dense at the periphery; in the center it is traversed by radially elongated vacuoles. As the mother cell enlarges the peripheral cytoplasm also becomes vacuolate. The fewer and fewer starch grains are located in the corners of the meshes of the network of cytoplasm.

The mother cell wall at this time is approximately 0.5–0.6 μ in thickness, staining faintly with light green and remaining colorless after the use of methylene blue or of the triple stain. As the mother cell enlarges, a thin dark layer appears on the inner surface of this colorless wall. Within this in turn there develops a third layer which gradually becomes stainable with orange G. This third layer acquires a thickness of about 1 μ in typical spore mother cells of 70–80 μ diameter, and of 1.2 μ in those of the sporophytes from clone

[2] Apparently some nutritive cells enlarge more rapidly and for a longer time than others, for in a single capsule small, several-celled as well as large one- and two-celled nutritive bodies may be seen (fig. 20). As late as 32 days after fertilization, undivided or just divided nutritive cells only slightly smaller than the spore mother cells have been seen. A mitotic spindle was observed in one of these. The spore mother cells in the capsule in which this dividing nucleus of a nutritive cell was found have large resting nuclei and are not ready to divide; each of the smaller nutritive bodies is composed of several cells.

30.613, a separable female. This innermost layer and the thin, more darkly staining layer are usually easy to distinguish. The unstained outer layer can be seen definitely only when a section of a mother cell slips and comes to rest on some other section. In figure 24, a photograph of a spore mother cell, the outer layer of the mother cell wall, transparent to the eye, shows a definite boundary. At this stage, therefore, the mother cell wall consists of three layers. These may still be seen around the young tetrads shown in figures 4, 5, and 11. The three-layered wall breaks away from the protoplast as a unit when the mother cell is plasmolyzed.

The nucleus enlarges as the mother cell grows. In a spore mother cell with a diameter of 45 μ there is a nucleolus, staining very deeply with safranin or haematoxylin, whose apparently rough outline is due to the concentration about it of strands of the chromatin-linin network. The rest of this network is distributed throughout the nuclear cavity. In a mother cell 60 μ in diameter the chromatin-linin network is more sharply defined. The nucleus at this stage and also at somewhat later stages is uniformly excentric in position within the mother cell (fig. 24). In mother cells of a diameter of about 65 μ, in a capsule of a sporophyte from the female clone 30.613, an interesting configuration appears. A large nucleolus containing a clear vacuole lies at one side of the nucleus; a smaller red-staining body lies beside the nucleolus. A number of very small red-staining bodies are seen on the slender strands of the chromatin-linin network which lie about the nucleolus. In mother cells of a diameter of about 80 μ the nucleus is in diakinesis and centrally located. The bivalent chromosomes occupy one side of the nuclear cavity. Figures at this stage are similar in both types of mother cells.

Just before their division the spore mother cells have a very characteristic appearance. In triple-stained preparations the vacuoles show as radially elongated areas in the gray cytoplasm. A few starch grains, pale blue with red centers, are still present. These contrast sharply with the dark blue starch grains in plastids in the nutritive cells and in the cells of the capsule wall. A translucent zone of cytoplasm lies just inside the cell wall. The mother cell stains deeply and rapidly with iron-alum haematoxylin at this stage and destains slowly, while the nutritive cells and capsule wall destain rapidly.

Thus far no distinction has appeared between spore mother cells

that are to give rise respectively to separable and to adherent spores, unless it be the somewhat thicker inner wall layer in the former.

Division into spores

LORBEER (**21**) found in his sporophytes of *S. donnellii* that division of the mother cells occurred 42 days after flooding of the parent plants. In my material division occurred from 30 to 34 days after flooding. However, as LORBEER found, within a given capsule the condition is fairly constant for all mother cells. Young tetrads occur in material fixed as early as 30 days after fertilization (sporophytes of females 29E3 and 30.613 of the separable races), while undivided mother cells are found in some capsules of either type fixed 34 days after flooding. The time of occurrence of the reduction divisions undoubtedly varies greatly with growing conditions.

My preparations show only a few of the stages in meiosis. The nuclear appearance in spore mother cells of various sizes up to the time of division has been briefly outlined in connection with the description of mother cells. Diakinesis was observed in a 33-day separable and in a 34-day typical capsule. The bivalent chromosomes lie close together in most nuclei that I have observed, probably before actual diakinesis. In some nuclei the chromosomes are spread out so that the bivalent structure may be observed, and as many as seven pairs have been counted in one such nucleus. These chromosomes are granular and somewhat larger than those seen later on the spindle. In these same capsules occur several spindles of the heterotypic division, two doubtful cases of the binucleate stage, and in one capsule a single tetrad.

In the 34-day capsule from typical female 31.453, in which most nuclei are in early diakinesis, a spindle of the heterotypic division was seen. Five chromosome pairs are in metaphase; the other three pairs are not present. In the 33-day capsule from separable female 30.613, an anaphase of the heterotypic division was seen. Three separated pairs of chromosomes in one section and four pairs in the next succeeding section account for the seven somatic chromosomes of the haploid complement, but the X-Y pair is lacking. LORBEER (**21**) found that the X-Y pair separate before the others and pass to the poles first. This may account for their absence in both the

anaphase and metaphase figures just described. In the anaphase at least one or two of the chromosomes passing to the poles appear double in structure.

No cell plate was seen after the first division. In the two binucleate mother cells observed no division of the cell had occurred. After the homoeotypic division, the mother cell is divided by cell plates (presumably) into four protoplasts, which lie within the mother cell wall (figs. 11, 21). Fibrous cytoplasm with strands perpendicular to the separating plasma membranes is a characteristic feature of newly formed spore tetrads in the separable race (fig. 11), and of the (usually rare) dyads in the typical race. In the dyads this fibrous cytoplasm occupies a broad spindle-shaped area between the two nuclei. In young tetrads of the typical race the fibrous cytoplasm is not very noticeable. A young spore has a large nuclear cavity in which the chromatic material is aggregated toward one side (figs. 11, 21).

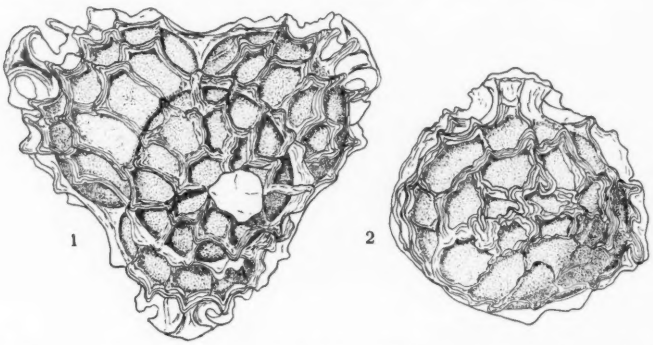
The development of the tetrads or separate spores from the spore protoplasts within the mother cell wall may best be considered separately for each type. The development of the typical tetrad (fig. 1) has been traced in the sporophytes from the mating 31.453×31.745, supplemented by those from the mating 23.107×31.1021. Similarly, the history of the separate spores (fig. 2) has been followed rather fully in the sporophytes of 30.613×31.773, and careful comparison has been made with development in the sporophytes of the other matings of separable females (30.613 and 31.453 were the only females which produced enough sporophytes to allow daily fixation during the critical periods).

Development of walls of typical tetrad

After the division of the spore mother cell the four spore protoplasts lie within the mother cell wall, usually in such a position that an approximately median section of the spherical tetrad shows three spores as about equal sectors of a circle (fig. 21). No middle lamella is laid down between the spores, but very early there may be observed a deposition of material between the outer, slightly rounded angles of the protoplasts and the mother cell wall, like a very blunt wedge. Patches of this same material appear on the inner face of the mother

cell wall against the outer faces of the spores (fig. 3). This is the beginning of what will here be called the "special wall," the term used by GATES (**12**) in describing pollen-tetrad wall formation in *Lathraea*. It is equivalent to LEITGEB'S (**19**) "special mother cell wall" and to STRASBURGER'S (**26**) "thickened mother cell wall," but is a layer separate from the mother cell wall proper.

The special wall material is probably pectic in nature, since it stains deeply with methylene blue, in striking contrast, when it



FIGS. 1, 2.—Fig. 1, typical tetrad of *S. donnellii* showing ridges and characteristic protuberance on middle of outer face of each spore; fig. 2, lateral view of separable spore showing small dorsal protuberance and ridges more pronounced on dorsal (outer) face than on ventral. ×368.

first appears, to the almost unstained mother cell wall (fig. 3) whose thin dark median layer seems to be its outer limit. In some cases all three layers of the spore mother cell wall are distinguishable (figs. 4, 5). The special wall stains with orange G; therefore, in triple-stained preparations the mother cell wall can be distinguished only because it has a discernible inner boundary. Treated with light green, the special wall is bright green, and the mother cell wall has a dull green inner layer and a distinct yellow outer layer.

The cushion-like patches of the special wall become thicker, reaching a thickness of 5 or 6 μ in the middle of each patch. Between these masses there remain depressions, arranged in the pattern of the future wall markings. Each spore protoplast follows the contour of the special wall on its dorsal (outer) face, extending to the tip of

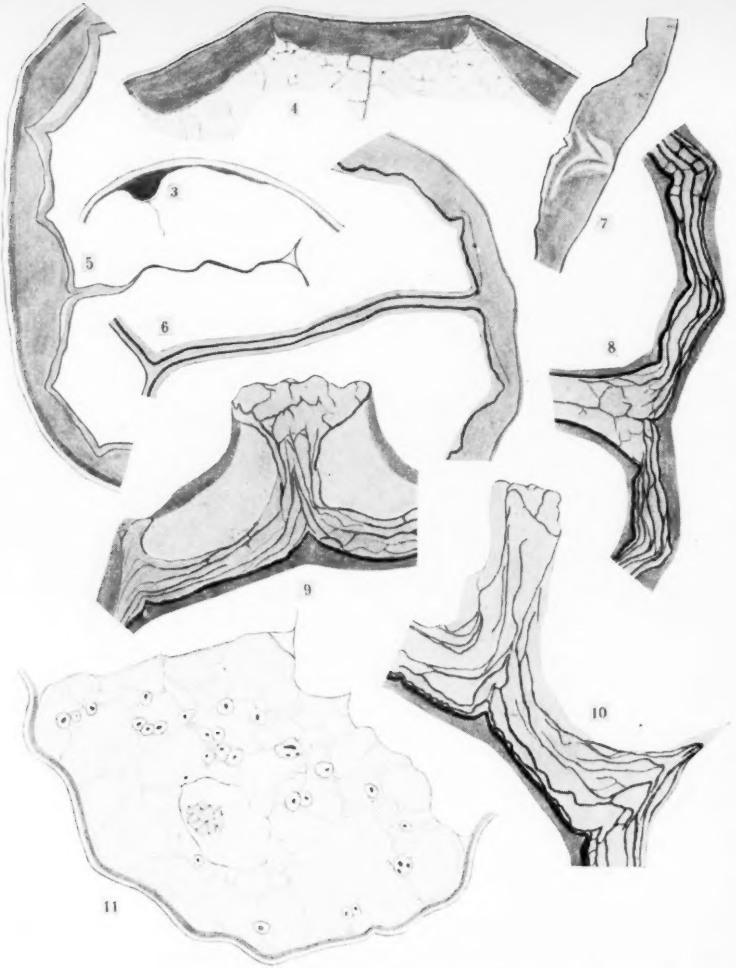
each indentation (fig. 4). The protoplasts are tightly pressed together at their outer angles and there is as yet no wall between their inner faces (fig. 4). Viewed as a flat expanse, the inner surface of the special wall would present a network of furrows, while the dorsal faces of the spore protoplasts would project in a network of ridges, fitting the special wall as a cast fits its mold. The special wall is actually a thick layer deposited by and incasing the tetrad of spore protoplasts which as yet have no complete walls of their own.

Soon, however, each spore protoplast deposits a thin layer completely around itself. As this is the first wall laid down around each spore of the typical tetrad, it will be spoken of as the "first spore wall." This layer when newly deposited stains red-violet with the triple stain, and on the dorsal face of each spore shows distinctly in contrast to the orange-staining special wall, whose contour it follows and to whose inner face it adheres tightly.

The walls along the inner faces (the planes of contact of the spores) now form the first septa between the spores. They lie close together at first, but almost immediately after their formation (fig. 5) a small amount of special wall material appears between them at the outer margins of adjacent spores. Almost simultaneously with the deposition of the first spore wall, the inner part of the special wall (which continues to grow thicker over the outer faces of the spores) shows differentiation into a less dense layer that follows the contours of the new first spore wall, adheres closely to it, and tends to break away from the rest of the special wall (fig. 5). This inner layer of the special wall is continuous with the special wall material of the septa between the inner faces of the spores, suggesting the origin of this latter material by an extension or flowing in of part of the less dense layer. The special wall material finally forms a thin layer between the first spore walls of adjoining spores (fig. 6).

The first spore wall increases in thickness by further deposition. It stains very darkly with methylene blue and is probably of pectic nature. After reaching its maximum thickness it is stained by safranin in triple-stained preparations.

The outer part of the special wall is easily separated from the inner thin special wall layer which immediately surrounds the tetrad. Sections of tetrads fixed at this stage sometimes lose the outer part



FIGS. 3–11.—Figs. 3–10, development of typical tetrad: fig. 3, first appearance of special wall as irregular deposit inside spore mother cell wall on dorsal face of a spore and between margins of two adjoining spores; fig. 4, adjoining margins of two spores showing 3-layered mother cell wall and indented special wall surrounding tetrad, cytoplasm extending to tips of depressions; fig. 5, wall between two adjacent spores and part of their dorsal walls, showing 3-layered mother cell wall, thick special wall over dorsal faces with first lamella and less dense layer just outside first spore wall, and a small amount of special wall material between first spore walls at outer margins; fig. 6, margins of adjoining spores showing differentiation of first lamella outside first spore wall, special wall material completely separating first spore walls on ventral faces, and newly deposited intine; fig. 7, portion of thick special wall on dorsal face of spore after deposition of first spore wall, showing localization of stainable material accompanying differentiation of the lamellae which are beginning development of dorsal protuberance; fig. 8, margins of adjoining nearly mature spores showing intine complete, heavy first spore wall, lamellate layer with four or five lamellae, and undifferentiated special wall material to outside, the mother cell wall being no longer distinguishable; fig. 9, dorsal prominence of nearly mature spore with wall layers same as in fig. 8 except that lamellae have developed outward at angle to build up the prominence; fig. 10, mature dorsal protuberance and prominent dorsal ridge of spore of typical tetrad showing no undifferentiated special wall left and mother cell wall disappeared; first spore wall is heavy and intine thick. Fig. 11, spore of newly formed separable tetrad showing spore mother cell wall that incloses the tetrad, starch grains in cytoplasm, and nucleus with excentrically placed chromatic material. ×936.

of the special wall, then consisting of the tetrad and inner part of the special wall only. The tetrad with the inner part of the special wall may fall out of the sections, leaving only the outer part of the special wall on the slide. STRASBURGER (**26**) found a similar condition in *S. terrestris*.

At the outer limit of the special wall layer which has become differentiated next to the first spore wall a dark layer appears, as though stainable material had there condensed; this is the first lamella of the lamellate zone which will eventually be differentiated from the special wall. The first lamella parallels the contours of the first spore wall across the dorsal face of each spore, and turns inward between adjoining spores. It does not develop farther inward than the margins of the spores. This lamella is violet in triple-stained preparations.

The thick special wall around the tetrad continues to differentiate a succession of lamellae, apparently by localized processes of condensation outside the first lamella. That the lamellate structures belong strictly to the spores rather than to the tetrad as a whole is shown by sections through the margins of adjoining spores (fig. 8). No one lamella continues unbroken across the gap between spores. Each turns inward around the spore to which it belongs and either ends bluntly or adheres to another lamella. Consequently the ridges of adjacent spores in a tetrad (fig. 1) are contiguous but not continuous.

The lamellate system is not composed of five or six unconnected sheets of material, one inside the other, but is a coherent structure (fig. 8). Although certain of the lamellae stand out distinctly, close examination shows that these, if not adherent to one another, are interconnected by smaller thin lamellae. The lamellae lie within a matrix of lightly stainable material which includes the first less dense layer immediately outside the first spore wall.

At the time of differentiation of the first lamella, in the thicker region of the special wall on the mid-dorsal face of each spore are found the beginnings of the prominent dorsal protuberances and ridges of the mature tetrad. In sections through this region in preparations stained with methylene blue (fig. 7), alternate layers of less and more deeply stained material are seen to lie at various angles to

the first spore wall, instead of parallel to it. The denser of these layers are the first of the outward-projecting lamellae which build up the prominence on the dorsal side of each spore, and correspond to about the inner two lamellae of the protuberance in figure 9. The dorsal prominence is built higher by successive lamellae as the differentiation of the lamellate layer progresses (figs. 9, 10).

The ridges on the dorsal face of each spore become higher with the addition outward of each new lamella. As the tetrad matures, gradual drying out of the lamellate layer probably serves also to accentuate the ridges. The undifferentiated portion of the special wall becomes more and more reduced as successive lamellae are formed. Finally the faint remains of the mother cell wall (or of the special wall) are seen around the tetrad, extending from one ridge to the next. Where the lamellate layer dips in slightly between spores this outermost vestige is easily detected, stretching straight across from spore to spore. In most cases it is undoubtedly lost as the tetrad dries out at maturity, but in some mature tetrads it is still recognizable.

The special wall material between spores condenses irregularly and forms a cementing layer. This is thinnest between the middle portions of the inner faces and thickest between the apices and between the outer margins of adjacent spores (fig. 23). A fibrous structure is evident, especially between the spore apices (fig. 22). Occasionally in mature tetrads there is no condensed special wall between the spore apices (at the center of the tetrad), but an empty space (fig. 23). In such tetrads, however, the fibrous condensed material between the inner faces of the spores is regularly present just as in tetrads with material at the apices. Between the inner faces of adjacent spores the condensation has taken place in relation to each spore; in consequence, a thin, comparatively even sheet of condensed material marks a median boundary in each septum between two spores. In sections this sheet appears as a line traversing the middle of each septum (fig. 23).

An inner layer, the intine or endospore, is deposited by each protoplast during differentiation of the lamellate layer (fig. 6). It fills in the depressions on the inner face of the first spore wall, and has a smooth inner surface. It attains a thickness of about 6 μ.

The walls of a mature adherent tetrad then consist of:

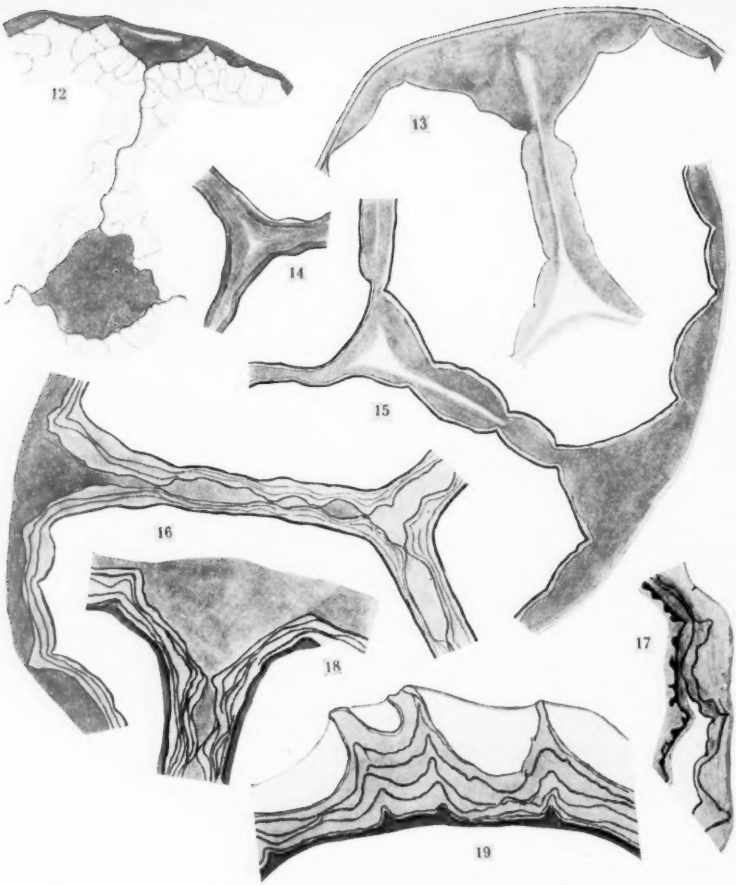
A. Two layers completely surrounding each spore:
   1. A thick intine whose inner surface next the spore protoplast is smooth and whose outer surface is ridged.
   2. The dark-staining first spore wall which fits the intine like an outer shell.

B. One or two layers surrounding the tetrad:
   1. The lamellate layer, which is only a thin, irregularly condensed region between the spores but is well developed over the dorsal faces, forming virtually a thick envelope for the tetrad, marked by prominent ridges and by a conspicuous tubercle in the middle of the dorsal face of each spore.
   2. Remains of the mother cell wall, if present.

Development of walls of separable spores

In young tetrads destined to become separated, the deposition of special wall material first becomes apparent, as in the typical tetrads, between the outer margins of the spore protoplasts, wedging them apart (fig. 11). This material is distinct from the mother cell wall which incloses the four spores. Along the inner faces of the spores the cytoplasm is fibrous, a condition which might be correlated with the secretion of special wall material by these regions, for special wall material is next deposited between the spores at their apices, in the center of the tetrad (fig. 12). At the same time the special wall masses on the dorsal faces of the spores thicken locally (fig. 12) just as in the typical race, retaining a network of depressions, the forerunners of the spore markings. The deposits between the spore margins increase greatly, becoming large wedges between them and connecting with the special wall material that is being deposited along the inner faces (fig. 13).

The special wall between spores arises as a layer external to the plasma membrane of each inner face. The material between the spore apices spreads out to contribute to this part of the special wall. Depressions of lesser magnitude than those on the dorsal faces are formed in the special wall between the spores (fig. 13), into which depressions the spore protoplasts extend. The indentations of



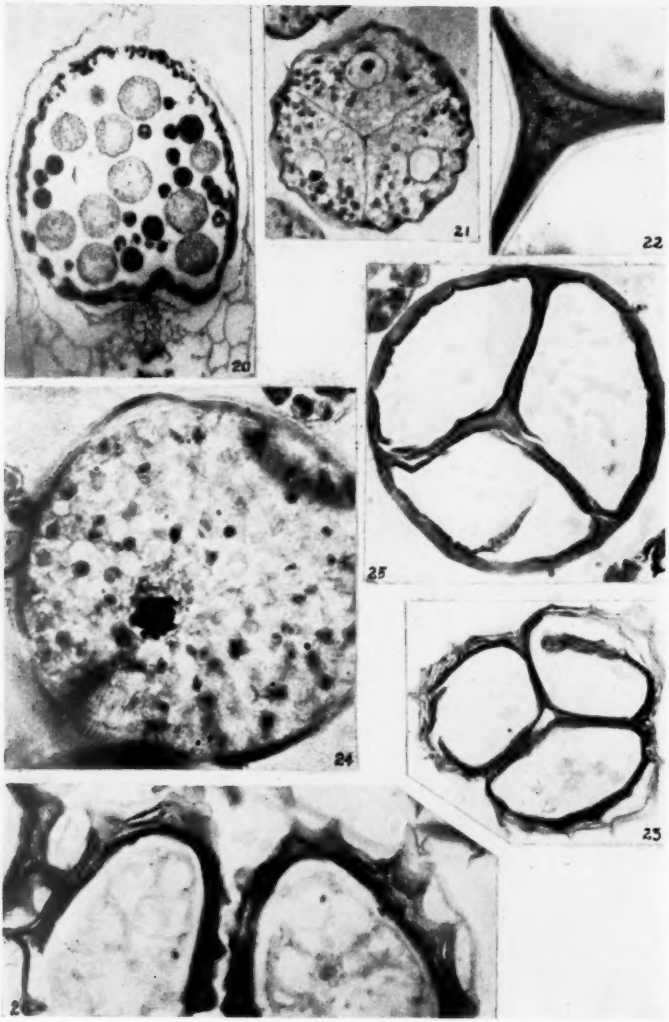
FIGS. 12–19.—Development of separable spores: fig. 12, formation of special wall in cushion-like masses inside mother cell wall and between apices of spores; fig. 13, margins of adjoining separable spores showing special wall between inner faces of adjacent spores with separating clear space extending into the bulge between spore margins, mother cell wall, and deposition of first spore wall as granular material; fig. 14, apices of three separable spores showing differentiation of first lamella in special wall just outside first spore wall; fig. 15, differentiation of first lamella in a tetrad about which the mother cell wall may still be distinguished; fig. 16, portions of two spores showing about three lamellae differentiated from special wall, and dark first spore wall; fig. 17, section of dorsal wall of spore showing from outside in: spore mother cell wall, undifferentiated special wall, two lamellae, first spore wall with irregular deposits of material on inner face, and intine; fig. 18, margins of two spores showing large wedge of special wall between spore margins, about five differentiated lamellae, the heavy, comparatively smooth first spore wall, and intine; fig. 19, section of dorsal face wall of mature spore showing small protuberance built up by lamellate layer outside the first spore wall and intine. This is part of wall of spore shown to right in fig. 26. ×936.

adjacent spores are opposite each other, the protoplast projections thus coming close together at these points.

While the inner special wall is being laid down between the spores it is definitely double. A clear space, dividing it, extends between the two separate deposits almost over the whole of the inner faces (fig. 13). A broader space between the spore apices is also noticeable. Staining reactions show that this is an open space, although probably exaggerated by fixation. Orange G, light green, methylene blue, and ruthenium red all stain the pectic special wall material and leave unstained this clear region, which corresponds in position to a middle lamella. A series of successively older spores show that this space cannot be interpreted as a split progressing from the center outward. The space, at first extending almost to the mother cell wall (fig. 13), becomes limited more and more to the center between spore apices (figs. 14, 15, 25), and finally disappears as the undifferentiated special wall material becomes uniformly distributed between the spores (fig. 16).

During and after the formation of the special wall, the young tetrads destined to become separated are easily distinguishable from those which will remain permanently united in tetrads. In the latter the special wall is developed as a tetrad-inclosing layer, appearing later between the first spore walls of adjacent spores as thin septa. In the separable tetrad the special wall completely surrounds each spore (fig. 25) even before the first spore wall is laid down. It is especially thick between the outer margins of adjacent spores. The mother cell wall, outside the special wall, surrounds either type of tetrad.

The special wall increases in thickness as the tetrad grows, but does not become so thick around separable tetrads as around typical ones. Within the special wall which surrounds each spore the protoplast deposits a layer corresponding with the first spore wall of typical tetrads (fig. 13). Because of its correspondence it will here likewise be called the first spore wall, even though the special wall is actually the first wall layer completely inclosing each spore. The first spore wall follows the contour of the special wall, and is therefore irregularly ridged on the inner faces of the spores and more regularly ridged on the outer faces. This new layer is at first thin, growing thicker,



FIGS. 20–26.—Figs. 20–23, typical race: fig. 20, longitudinal section of capsule containing young tetrads and nutritive bodies, cells of capsule wall containing many dark-staining starch grains. ×72; fig. 21, young tetrad from center of capsule shown in fig. 20. ×312; fig. 22, apices of three spores showing irregular condensation of special wall material, each spore with its own intine and first spore wall. ×880; fig. 23, nearly mature tetrad showing intine, first spore wall, and lamellate layer; nature of septa and junction of spore margins plainly shown. ×312. Figs. 24–26, separable race: fig. 24, spore mother cell showing three layers of wall and excentric nucleus. ×880; fig. 25, differentiation of first lamella in special wall outside first spore wall in young separable tetrad; note broad septa in comparison with fig. 9. ×468; fig. 26, mature spores still lying in tetrad, held together only by mother cell wall stretching from ridge to ridge and from spore to spore. ×468.

probably by deposition from the protoplast, as is suggested by the granular appearance of its inner surface (figs. 13, 25). Figure 17 shows a later stage at which the first spore wall has still an irregular inner surface.

The thickening of the first spore wall is accompanied by a differentiation of the special wall, similar to that which occurs in a typical strain. In a separable race, however, the lightly staining inner layer of the special wall and the condensing lamella surround each spore completely (figs. 14, 15, 25). Figure 15 shows the spore mother cell wall persisting, but all sections do not show it. Preparations stained in methylene blue are most favorable for observing the origin of the lamellae. The special wall where it is in contact with the first spore wall seems to become less dense. At the outer boundary of this less dense zone a condensation of stainable material occurs to form the first lamella. Outside the first lamella another pale (less dense) area may be observed.

The tetrad has been enlarging during the period of wall formation. Its growth has served to straighten out the indentations in the special wall and the first spore wall of the ventral (inner) faces (*cf.* figs. 15 and 16). The latter retain none of the ridges that were laid down in the depressions of the special wall. As the spores grow larger and older, the rounded portions of the first spore wall between the ridges on the dorsal face are also in considerable measure flattened out (*cf.* figs. 15 and 19, figs. 25 and 26).

The special wall on the ventral spore faces shows evidence of lamination earlier than does that on the dorsal faces. In section it has a stringy appearance. While only the first lamella is being differentiated about the dorsal side of each spore, several lamellae may be observed in the ventral faces of the special wall. The ridges of the spore-wall pattern are not marked at first in these lamellae, but as the lamellae become more definite the ridges become more apparent (fig. 16).

The bulge of special wall separating the margins of the dorsal faces of the spores is thickest where three spores abut. There is no marked thickening of the special wall over the mid-dorsal face of each spore as in the typical tetrads; neither is the differentiation of a large dorsal prominence on each spore evident.

After the appearance of the first lamella, more lamellae are differentiated from the special wall completely around each spore (figs. 16, 18). This occurs presumably in the same manner as the differentiation of the first lamella, that is, by alternate condensation and dilution or other modification of special wall material. The lamellae are not separate sheets; they adhere in places or are interconnected by thinner incomplete lamellae. The less stainable material of the special wall, as in the typical tetrad, forms a matrix inclosing the lamellae.

When about three or four lamellae are visible in the special wall around each spore, the intine becomes apparent. As in typical races it fills the hollow ridges of the first spore wall and has a smooth inner surface. The number of lamellae present on the outer face when the intine first appears seems to vary from as few as two (fig. 17) to four or five (fig. 18).

The lamellae follow the contour of the first spore wall, repeating each ridge and broad area between, thus carrying outward the spore-wall pattern. The ridges become a little higher in each new lamella. On the dorsal face of each spore the ridges become higher than on the ventral faces. With an increase in the number of lamellae the mid-dorsal ridges become more prominent than the others, and build up a much lower tubercle (fig. 19) than is characteristic of the typical tetrad. Outside the lamellae may be seen an ever-decreasing layer of undifferentiated special wall around the tetrad and between the spores.

The lamellae of the walls on the ventral faces acquire ridges. These are about as frequent and are situated at approximately the same locations as the original indentations of the special wall and the ridges of the newly deposited first spore wall (*cf.* figs. 15 and 26). The ridges of the first spore wall become obliterated on the ventral faces as the spores grow and the already formed wall layers stretch to cover the increased volume. The first lamella preserves a slight impression of the first pattern and this becomes accentuated by lamellae differentiating later, until a pattern of ridges less prominent than those on the dorsal face is built up on the ventral walls.

As the lamellate layer is differentiated, the thick bulge between spore margins (figs. 15, 16) becomes poorer in stainable material. As the spores round up, their edges are pulled farther apart. Finally,

when the lamellate layer is complete, there remains only a faint vestige of special wall or spore mother cell wall holding the spores in the tetrad arrangement (fig. 26). There is no way of ascertaining which layer this last inclosing wall represents; it is most probably the mother cell wall, for if part of the special wall becomes lamellate, all of it might be expected to be similarly changed. The last-persisting tetrad-inclosing wall is usually lost as the capsule and spore walls become dry at maturity. The spores are then free separate spores. Sometimes, however, the mother cell wall still holds mature spores together in tetrads. Mechanical pressure such as is exerted upon tetrads being mounted on a slide is sufficient to break this outer wall and to free the spores.

The drying of the ventral wall layers is also instrumental in separating the spores. In immature tetrads (fig. 16) the tips of ridges of opposed ventral walls appear to be adherent. In more nearly mature spores (fig. 26) the ridges no longer touch one another, and the vestige of spore mother cell wall is the only structure holding the spores together. The height of these ridges and the depth of the depressions between have increased, and the tips of the ridges have been pulled apart.

The layers in the wall of a mature separable spore (fig. 26) are therefore: (1) The intine, with smooth inner surface and ridged outer face. (2) The first spore wall, a thick, dark-staining layer, granular on its inner surface, smoother on its outer, and fitting like a shell over the intine, its ridges forming a basis (on the dorsal face at least) for the spore markings. (3) The lamellate layer, composed of five or six lamellae in a less stainable homogeneous matrix, in which from innermost to outermost lamella the ridges of the first spore wall are repeated with increasing prominence. The lamellae show greater individuality with fewer cross-connections than do those in the corresponding layer of the typical tetrad.

Discussion

Special wall

The terminology encountered in descriptions of dividing spore mother cells is somewhat confusing because of its origin in studies made before the recognition of the true nature of cells. The cell to early workers was the wall; hence the thick gelatinous wall surround-

ing and inclosing the four parts of a divided pollen mother cell were special mother cells (**25**). After the true nature of cells was better known, the term special mother cell was transferred to the newly formed spore protoplasts themselves, and the gelatinous wall was called the special mother cell wall, as in the writings of STRASBURGER. LEITGEB (**19**) used the term special mother cell for either the spore protoplasts or the gelatinous walls surrounding them. PETOUNNIKOW (**23**), using the old terminology, designated the walls as the special mother cells. As late as 1906, BEER (**4**) speaks of the spore protoplasts as special mother cells, and of the walls between them as primary and secondary special mother cell walls (equivalent to the terms middle lamella and special wall as used in the present paper).

BENSON (**5**) has pointed out the absurdity of retaining the unnecessary and erroneous term "special mother cell." The gelatinous wall may still, I think, be called a special wall, since this combination of words is easily identified in older and also in more recent descriptions of pollen and spore walls with structures similar in development.

TYPICAL TETRADS

Comparison of the present description of the development of the wall layers of the persistent spore tetrad in *Sphaerocarpos donnellii* with previous descriptions of the wall development in *S. terrestris* immediately presents some points of difference.

In *S. donnellii* the spore mother cell divides while surrounded by only the comparatively thin mother cell wall, and young tetrads are found inclosed only by this wall. Later the thin mother cell wall of uniform thickness can usually be seen outside the thickening masses of the special wall, which is apparently a new layer deposited by the spore protoplasts. The plane of demarcation between adjacent spores is now formed only by the thin plasma membranes of the ventral faces of the protoplasts. The septa, that is, the wall layers between spores, in a typical tetrad are not formed until after the special wall is well developed around the whole tetrad. Lightly stained preparations of this stage at which the special wall surrounds the tetrad give the impression of being undivided thick-walled mother cells unless the preparation is examined critically.

Both LEITGEB and STRASBURGER reported that in *S. terrestris* the indented special mother cell wall is formed around the mother cell protoplast before the mother cell divides to form the spores. STRASBURGER'S (**26**) observations seem to have been made largely upon mounts of whole tetrads. In a young tetrad like that shown in figure 20 the presence of four spores would probably not be detected in a whole mount, especially after clearing with glycerin. Whether the spore mother cell of *S. terrestris* divides before or after the special wall appears cannot be stated without re-investigation of that form.

According to LEITGEB (**19**) no middle lamella is laid down between the spores in *S. terrestris*. STRASBURGER saw a middle lamella in each septum between spores of a mature tetrad of the same species. In *S. donnellii* no middle lamella has been distinguished.

There is evidently a differentiation in *S. terrestris* similar to that which has here been described as occurring in the special wall of *S. donnellii* immediately after the beginning of the deposition of the first spore wall. In the latter species this is plainly a differentiation of the inner portion of the special wall on the outer faces of the spores, immediately following the appearance of the first spore wall. In *S. terrestris* both LEITGEB and STRASBURGER observed this differentiation before the separation of the spores by visible septa. They reported that this innermost layer of the special wall is differentiated around the mother cell. STRASBURGER interpreted it as a wall layer deposited upon the special wall by the mother cell protoplast; therefore he called it the outer exine. The evidence was his observation that the ridges of this inner layer do not reach to the tips of the indentations of the special mother cell wall. He also stated that if a layer were differentiated from the special mother cell wall with its papillate projections, it would not be a coherent layer but would be composed of separate pieces of material. LEITGEB (**19**) had previously reported that a thin layer and a thicker peripheral layer are differentiated in the special mother cell wall, the inner layer being the forerunner of the perinium which surrounds the mature tetrad.

Observations on *S. donnellii* agree with LEITGEB'S interpretation of the layer in question, namely, that the first lamella and the pale layer inside it are differentiated within the special wall. STRASBURGER'S

first argument may be answered by the fact that in *S. donnellii* the inner layer of the special wall adheres more tightly to the first spore wall than to the outer undifferentiated portion of the special wall (fig. 5). His second objection would not hold in this species. Here either the special wall continues to grow in thickness or the material is always being redistributed, for as each lamella is differentiated the tops of its ridges extend out to the mother cell wall, just as did the depressions in the special wall when first laid down. The lamellae and layers inside them are continuous, and are not composed of separate pieces divided by the original furrows or indentations of the special wall, as STRASBURGER inferred would be the case.

The late appearance of special wall material between the spores in *S. donnellii*, after the deposition of the first spore wall, may be partially correlated with LEITGEB'S observations in *S. terrestris* (**19**) that the daughter nuclei move apart and the septa between spores are laid down, after which each spore deposits a membrane of its own. STRASBURGER says that the wall separating the spores becomes continuous with the outer exine, after which the inner exine is laid down around each spore. STRASBURGER'S inner exine, when first laid down, and LEITGEB'S membrane around each spore, correspond to what has here in *S. donnellii* been called the first spore wall.

LEITGEB says that the inner lamellate portion (perinium) of the special mother cell wall increases in thickness as the outer layer loses its massiveness; the outer layer is finally reduced to a peripheral lamella that eventually disappears. This process in *S. terrestris* corresponds almost exactly to the differentiation of the lamellate layer from the special wall in *S. donnellii*. The spore mother cell wall which is left around the tetrad is equivalent to LEITGEB'S "peripheral lamella."

STRASBURGER'S account of the maturation of the wall layers is different. According to him the inner exine is the layer that becomes lamellate, and the outer exine, which forms a complete layer around the outside of the mature tetrad, thickens to only a small extent. The special mother cell wall with its thickening masses is lost as the other wall layers grow. STRASBURGER'S outer exine corresponds to the outermost layer of LEITGEB'S perinium, while his inner exine includes the rest of the perinium plus the exine around each spore.

The terms exine and perinium have been avoided in the present paper. What they signify is largely an academic question. That the special wall is deposited originally by the spore protoplasts is evident in *S. donnellii*. When it first appears it might be called an exine. The first spore wall is an exine also, corresponding closely to the exine of many other spores. But the lamellate layer, developing as it seems to from the special wall, can hardly be called by the same name as the exines of the spores of *Riccia glauca* or of pollen grains, which, according to descriptions, involve little if any of the special wall.

Separable spores

The development of the separable spores must be compared with that of the separate spores of other hepatics as well as with that of the spores of typical tetrads in *S. donnellii*. In the few separate-spored hepatics for which any details of spore-wall formation have been given (see Introduction), the thick special wall between and around young spores has been noted. This is the case in *Riccia glauca*, the history of whose spore walls has been most fully studied. Beer (**4**) states that the secondary special mother cell wall of callose is deposited about each spore within the mother cell membrane and within the primary special mother cell walls between spores (the middle lamellae). No such middle lamellae were observed in the separable tetrads of *Sphaerocarpos donnellii*, although the special wall had the same distribution as described by Beer.

Leitgeb (**20**), to judge from Strasburger's statements, evidently interpreted the outermost layer of the spore wall of *Riccia glauca* as a perinium derived from an inner layer of the special mother cell wall. If this is the case, then the outer layer of the spore wall of *R. glauca* is comparable in origin with the lamellate layer of the separable spores of *S. donnellii*. Strasburger concluded that this outermost layer in *R. glauca* is an outer exine, deposited by the spore protoplast inside the special mother cell wall and therefore not a perinium. If this interpretation is correct, the outer exine of *R. glauca* is not comparable with the lamellate layer of separable spores of *S. donnellii*. Beer did not attempt to settle the question of the origin of this outer layer in *R. glauca* because of the insufficiency of his evidence.

The inner exine of *Riccia* in its mature 3-layered condition (**4**) differs from the single-layered first spore wall in *S. donnellii*. The spores of both forms have comparable intines.

The young tetrads of separable spores of *S. donnellii* resemble the young tetrads of *Riccia glauca* (**4, 20, 26**), *R. frostii* (**7**), *R. trichocarpa*, *Fimbriaria californica* (**10**), and *Reboulia hemisphaerica* (**14**) in the distribution of special wall material, that is, in the feature which apparently is associated with the separable spore character. However, the layers present in the mature wall and their development are features characteristic of *S. donnellii*, exactly comparable only with the corresponding wall layers and their development in the typical tetrad.

Basis of separate or adherent spore character

A comparison of the mature spore walls of the separable and nonseparable spored races of *S. donnellii* shows the only conspicuous difference to be in the distribution of the lamellate layer. In the separable tetrad this layer is of comparatively uniform thickness around each spore. In the adherent tetrad it is thick over the dorsal faces, with a prominent tubercle developed on each spore, but thin and irregularly condensed between spores so that it holds them together. The developmental history of the spore wall shows that the location of the lamellate layer of the mature wall is determined by the distribution of the special wall material of the young tetrad. If the special wall is deposited only on the dorsal face of each young spore protoplast, the spores will remain united in tetrads at maturity; if each protoplast becomes completely surrounded by the special wall, it will develop into a separable spore.

Spore wall pattern

A comparison of the number and distribution of the indentations in the special walls of tetrads of *S. donnellii* and *S. terrestris*, and of separate spores of *S. donnellii* and *Riccia glauca*, indicates a correspondence of these indentations with the ridges of the specific tetrad- or spore-wall markings which are diagnostic of mature spores or tetrads.

Leitgeb (**19**) ascribed the configuration of the wall layers deposited inside the special mother cell wall, and of the layers formed

from this special wall in *S. terrestris*, to the primary vacuolate structure of the protoplasm and to the swelling pressure of the persistent mother cell wall.

Certain advantages may conceivably be ascribed to an indented special wall. On the outer face of each spore with such a special wall the protoplast retains connection with the mother cell wall until the first spore wall is laid down. This condition may allow a more rapid intake of water and food materials. The first spore wall and the successively formed lamellae in turn each repeat this connection of their ridges with the mother cell wall, that is, with the outer limits of the special wall.

A papillate surface has a greater area than a smooth one; therefore a wall layer deposited over such a papillate surface would be better suited to cover an expanding body than a similar layer deposited upon a smooth surface. A simple mathematical computation demonstrates the advantage of the deposition of the first spore wall upon the series of approximately hemispherical thickening masses of the special wall.

A uniform layer of material deposited on the surface of small hemispheres on the inner face of a large sphere has about twice the surface of the large sphere, and hence can be expanded without increase in area to cover a sphere of twice the volume of the original one and with a diameter of 1.414 ($1\sqrt{2}$) times the original one. Measurements of typical tetrads at the time the first spore wall is deposited over the papillate surface of the special wall, and of similar tetrads when mature, show on the average an increase in the distance from apex to outer face of from 41 to 52μ, 1.29 times the original dimensions. The fact that the increase of the spore cavity is as slight as this seems to show that the first spore wall, once deposited, does not need to grow by intussusception to accommodate the growing protoplast. It is deposited originally with a surface area adequate to cover the protoplast of the mature spore, and may as a consequence be a limiting factor in the final size of tetrad or spore.

Summary

1. Spore mother cells of both typical and separable spore races of *Sphaerocarpos donnellii* develop in a similar manner, dividing to form young spore tetrads inclosed by a thin mother cell wall.

2. In a tetrad of a typical race, a gelatinous special wall, composed of hemispherical masses with depressions between, develops about the tetrad and later is extended as a thin layer between the spores. In a separable race the hemispherical masses of special wall develop also over the inner faces of the spores.

3. In both races there is deposited about each spore a darkly staining first spore wall with ridges corresponding to the depressions in the special wall, and a thick intine.

4. The special wall in either case becomes differentiated into a lamellate layer. In the typical tetrad the lamellate layer is thick over the dorsal faces of the spores, where it develops a prominent tubercle, and thin and irregular between spores, holding the tetrad together. The lamellate layer surrounds each spore of the separable tetrad on all faces, with a much less prominent dorsal protuberance.

5. Typical and separable tetrads differ at maturity mainly in the distribution of the lamellate layer. Thus the original distribution of special wall material determines whether the spores will remain united or will become separable as they mature.

6. The indented special wall, and the first spore wall following the contour of the special wall, allow for the expansion undergone by the spore protoplast during the maturation of the spore walls.

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PHLOEM ANATOMY IN TWO SPECIES OF NICOTIANA, WITH NOTES ON THE INTERSPECIFIC GRAFT UNION

A. S. Crafts

(WITH PLATES XI, XII AND NINE FIGURES)

Introduction

Recent studies on the movement of organic nutrients in plants (**6, 7, 8, 14, 15, 19**) indicate the important rôle of the phloem in this process. Numerous researches, however, have failed to develop a thoroughly satisfactory theory to explain the rapid flow of materials. Although the sieve tubes seem fundamentally involved, their exact place in the mechanism is yet to be determined. Even more recent studies (**1, 11, 12**) indicate that certain insect-borne viruses spread within the plant, after their introduction, through the phloem tissues. Here again the sieve tube apparently takes part in the process of translocation; and, on strictly anatomical grounds, its ability to conduct seems closely related to its ontogeny. In the sugar beet, internal symptoms of curly top are initiated relatively close to mature sieve tubes (**11**).

Bennett showed (**1**) in his ringing experiments that the causal agent in curly top could not pass a ring unless there was a bridge of vascular tissue. In grafts, he found that the curly top agent moved from one member to the other only after vascular continuity had become established. As two species of *Nicotiana* have played an important part in these studies, providing the material for the work on translocation, it seems justifiable to investigate in detail phloem development as related to the formation of the graft union and to conduction across it in these species. The present work describes phloem ontogeny in *Nicotiana glauca* Graham and in *Nicotiana tabacum* L. It indicates the nature of union in the interspecific graft, shows how rapidly the first vascular connections may be formed, and discusses phases of the process that should interest horticulturists in general.

Normal phloem anatomy

The phloem of *N. tabacum* will be described in detail, *N. glauca* being mentioned only when interspecific differences occur. Primary phloem of the stem, produced by differentiation of the procambium strands, consists of sieve tubes, companion cells, and phloem parenchyma. In the stage immediately following division of the sieve-tube mother cell, the young sieve-tube element is an elongated cell with transverse end walls, a heavily staining nucleus, and one or more slime bodies (figs. 1, 5). These bodies are often found in close association with the nucleus but not constantly so.

In the young sieve tube, the nucleus is well defined and contains one or more nucleoli. The slime bodies are homogeneous, ovoid, or spindle-form structures, definite in outline and easily distinguished. Although visible in unstained living cells, they may be more readily observed after staining with water-soluble anilin blue.

In the youngest sieve-tube elements there also occur small spherical particles that stain darker than the bulk of the cytoplasm. These appear to be the primordia of the characteristic sieve-tube plastids. They later become much more prominent.

After treatment with IKI and water blue, the protoplasm of the young sieve tube appears granular in structure, usually with one or more prominent vacuoles. In living tissues these vacuoles accumulate neutral red, and the protoplasm exhibits active streaming. With certain killing treatments the protoplasts of the cells are shrunken, the protoplasm readily pulling away from the side and end walls.

With increasing age the nucleus enlarges and decreases in density of staining; the nucleoli shrink and finally disappear (fig. 2). The slime bodies enlarge, assuming a more complex internal structure (figs. 2, 6). Their form changes, becoming longer, less dense, and more spindle-like. The plastids increase in numbers and size, while the vacuoles unite to occupy the whole central lumen.

As the sieve tube matures the nucleus enlarges further; the contents become roughly granular, then thread-like; the nuclear membrane grows thin and disappears, releasing the thread-like particles into the vacuole, where they disintegrate. The slime bodies, enlarging, assume a complex stranded structure and start to fragment,

usually from one end (figs. 2, 6), leaving the lumen occupied by a mass of thread-like material that tends to adhere to the internal protoplasmic strands. This material later breaks loose and, under the influence of certain killing agents, may be induced to accumulate at one end of the cell, forming the so-called "slime plug" (figs. 3, 7). In the normal living cell these thread-like strands are affected by protoplasmic streaming and thermal agitation to produce the peculiar motion previously described in potato (**7**). They slowly disintegrate, leaving within the cell an amorphous colloidal suspension, subject to the agents just mentioned and able to produce amorphous slime plugs (fig. 4). In the living tissue this colloidal material finally disappears, leaving no coagulable contents in the sieve tubes (fig. 8).

In the maturing sieve tube the protoplasm stains less densely than in the young element. It assumes a fibrous structure when stained, and takes principally a parietal position as the vacuoles unite. The plastids enlarge, are stained pink or reddish brown with iodine, and assume what appears to be a spherical form.

Meanwhile the sieve plate, which up to this stage has differed from other end walls only by an uneven staining of the middle lamella, begins to thicken; the protoplasmic strands that traverse it may be stained. Neutral red accumulation by living sieve tubes becomes less and less pronounced, while all streaming movements of the protoplasm cease. In the mature element the protoplasm forms a thin parietal layer, with a meshwork of very fine strands traversing the lumen and connecting the sieve plates and side wall pits. Vital stains are not accumulated, and plasmolysis cannot be accomplished even with concentrated salt or sugar solutions.

The small callus cylinders that eventually surround the protoplasmic strands of the sieve plate are initiated as the elements mature. First appearing as small collars located on opposite sides of the plate, they increase in length, uniting at the middle lamella and extending farther and farther into the lumina as the plate thickens. Finally they fuse laterally to form a perforated mass through which the greatly stretched protoplasmic strands extend. The plastids, attaining full size, leave the parietal protoplasm. Many adhere to the the inner meshwork of threads, while others apparently become free within the vacuole, exhibiting rapid Brownian movement.

In the petiole and leaf the phloem remains primary throughout the life of the plant, so that sieve tubes of the type just described may be found in various stages at almost any time. In the stem secondary growth soon displaces the primary tissues, and sieve tubes of a different type are found. Secondary sieve tubes are larger, on an average, than primary ones, being broader, usually somewhat curved in form, and having diagonally or longitudinally arranged sieve plates. The diagonal plates have one or more sieve fields; the longitudinal ones often have a greater number.

The slime bodies common to primary sieve tubes have not been found in secondary ones. Arising from cambium cells, the secondary tubes show also a different nuclear behavior. With the early differentiation of the element, the nucleus enters the prophase stage characteristic of mitosis, one or even two divisions rapidly following. The fully differentiated element contains from two to four resting nuclei, which very soon disintegrate, following essentially the same course as do those of the primary tube. A similar behavior has been noted in gymnosperms by STRASBURGER (**20**). Plastids are very prominent in secondary sieve tubes; and, accumulating carbohydrate, they remain the only inclusions characteristic of the mature element. The cytoplasm is principally parietal with a few strands traversing the lumen. Like the primary, the secondary tubes also lose their ability to accumulate vital stains and to be plasmolyzed; evidently they, too, become completely permeable with maturity.

In the stem of the growing or blossoming tobacco plant most of the sieve tubes are in the "mature stage" just described. All primary sieve tubes are finally obliterated, and young enlarging elements form only a very small fraction of the active phloem tissues.

In tobacco the protoplasmic strands of the sieve plate are extremely fine, varying from 0.2 to 0.1 μ or less in diameter. Starting with an initial length of 2 to 3 μ, they are stretched to many times this length as definitive callus is laid down. The parietal protoplasmic layer and the internal strands become extremely thin and may be demonstrated only by careful staining.

As the permeability of the maturing sieve tube increases, the elements lose turgor and can no longer maintain their form in competition with surrounding living cells. This condition is evidenced by

the slow pushing in of the side walls and the complete closing of the lumina with obliteration. As the contents are resorbed, nothing remains to mark the position of the tube except the thickened regions in the walls and occasional sieve plates crowded between phloem parenchyma cells.

The companion cells change but little with maturity of the phloem. Starting as narrow cells with dense cytoplasm containing small vacuoles, they increase slightly in size, the vacuoles enlarging somewhat. The protoplasm remains relatively abundant, retaining all the properties it possesses in normal living cells. It shows streaming movements, accumulates vital stains, and reacts normally to plasmolysis. Only at senility of the sieve tube does the companion cell degenerate and collapse, finally becoming obliterated with its sieve-tube element.

Phloem parenchyma consists of elongated cambiform or rectangular cells of approximately the same size as sieve-tube elements. Expanding rapidly as they mature, their vacuoles unite to fill their lumina; the protoplasts become parietal; chloroplasts attain their full development. In their mature form phloem parenchyma elements display all the properties of normal living cells. Upon obliteration of the sieve tubes, the surrounding parenchyma cells expand slightly to occupy the space. Certain phloem parenchyma cells are early filled with crystal sand. These persist through the life of the stem, and their numbers may increase in older plants.

In *N. glauca*, phloem ontogeny follows the same course as in *N. tabacum*, with no characteristic deviations in the various stages that would serve to distinguish the two species. Certain structural variations serve in differentiating the two, however, especially in the phloem of the graft union.

The pitting of the walls is more pronounced in *N. tabacum* than in *N. glauca*, a fact indicated in figures 1–8. In *N. tabacum* all phloem elements average slightly larger than in *N. glauca*, and the vascular rays are noticeably broader. In the xylem the vessels of *N. tabacum* are composed of short, relatively broad elements; those of *N. glauca* are noticeably longer and narrower.

Tobacco graft union

Many publications describe the formation of the graft union in fruit trees and in various other horticultural plants. The present study primarily concerns the establishment of vascular continuity in the union of the two tobacco species mentioned, and other works will be cited only if related to this problem.

WAUGH (**21**) pointed out years ago that the vascular tissues of stock and scion consist of fully differentiated cells, many of which are dead. To him it was obvious that these could not grow together. More recent works (**2, 3, 10, 13, 18**) clearly describe the intermingling of callus cells from the stock and scion, the establishment of a cambium layer within this callus tissue, and the development of vascular tissue in continuous sheets across the region of the union external to the woody cylinders of the original members.

The present studies on tobacco included both interspecific and intraspecific grafts of *N. tabacum* and *N. glauca*. The process of union formation was found to differ in several details from that taking place in trees. The interspecific grafts showed no signs of incompatibility and differed in no essential from the intraspecific ones. Unions of various ages were studied and grafts of several types were used. The only factor appreciably affecting the formation of the union was the matching of the scion and stock. The more closely the cambiums of these were matched, the more smoothly and rapidly was the union formed.

After the stem was cut in the procedure of grafting, there occurred a slight shrinking of pith, phloem, and cortex cells, with some browning of the cut surfaces. In many cases callus formation started within 48 hours. These grafts differed from those studied by SASS (**18**): callus was produced by division of parenchyma cells in the pith, phloem, xylem, and cortex, as well as by the cambium. Within a few days the entire surfaces of scion and stock were covered by a layer of callus tissue, which often completely filled any intervening spaces. In as few as five days after the graft was made, regenerative processes had started within the callus tissue; and after divisions in series of these cells, sieve tubes (fig. 9) and xylem elements (fig. 10) were simultaneously differentiated, connecting the younger vascular

tissues of stock and scion. Soon the mature xylem elements of stock and scion were filled with wound gum, while mature sieve tubes rapidly declined, becoming blocked with definitive callus, discolored where actually cut, and obliterated within a short time.

The regenerative stimulus seemed to emanate from the ends of young growing vascular elements, and only in continuity with the latter were the new connecting elements differentiated. When the vascular cylinders of stock and scion were well matched, the paths traversed by these connecting elements were short and relatively straight; when matching was poor, the paths were long and devious.

There seems to be no doubt concerning the origin of these connecting strands. In both phloem and xylem the cells are short, nearly isodiametric, and often oddly shaped. Their walls coincide with those of the surrounding callus parenchyma, from which they have apparently been derived by differentiation (figs. 9, 10). The sieve tubes have no slime bodies and resemble secondary sieve tubes of the stem in every way except form, while the xylem elements show the characteristics of secondary tissue.

By the time these connecting vascular strands are differentiated, cell division in the callus between these xylem and phloem elements gives rise to cambial tissue. By lateral extension, the cambiums of the strands unite to form a complete layer; and in cases where matching is close, a smooth sheet of tissue soon starts differentiation. Within two weeks a continuous layer may be formed, uniting the stock and scion. Development of this layer produces within the region of the graft an annual ring, differing in no essential feature from that of the stock or scion.

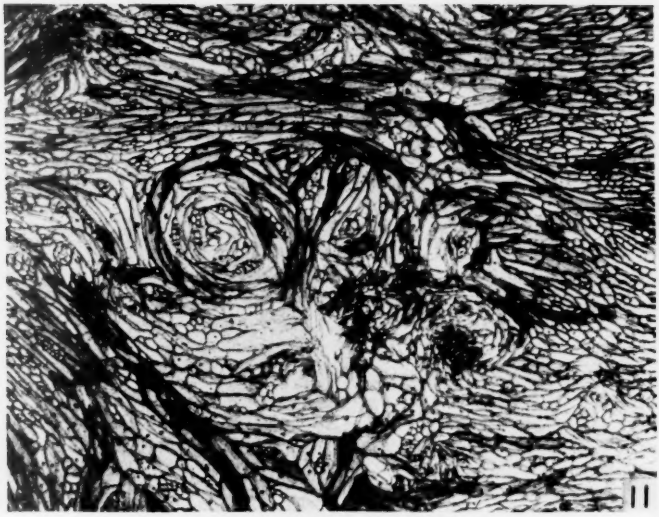
The orientation of the cambium initials produced within the callus is apparently determined by that of the vascular elements between which they lie. When the paths of the vascular elements are straight, the cambium arises as a smooth layer with longitudinally arranged elements of normal form. The vascular tissues, however, occur in strands. When these strands are very long and crooked, the cambium strips formed within them follow the same paths; and lateral connection between them must take place across broad masses of undifferentiated callus cells. The formation of the cambium layer is delayed, and when it is completed the initials lie in odd and varied

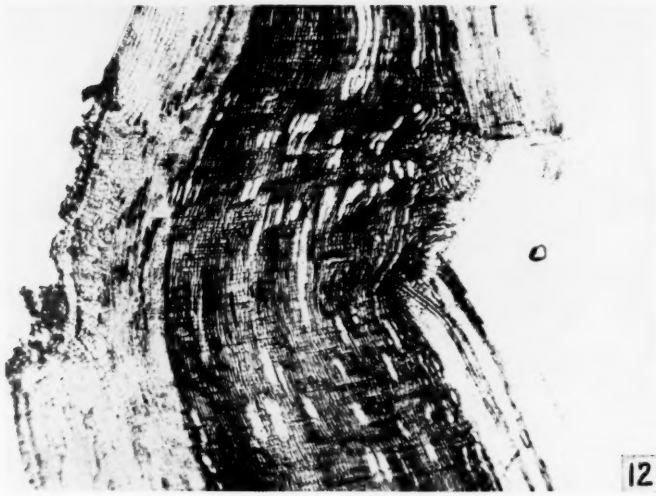
patterns. Vascular tissues differentiated from these initials assume the same pattern, as illustrated by the phloem in figure 11. When the matching of stock and scion is very poor, excessive amounts of this abnormal vascular tissue are formed and the graft union is rough. In those woody species in which there is no incompatibility, the cambium initials may slowly reorient and the graft may, within a year or two, become fairly smooth (**2, 18**). The strength of such grafts does not seem to be affected, and only when masses of callus or dead vascular tissue interrupt the cambium layer do the grafts remain permanently weak (**2, 17, 18**). In tobacco the graft may become rapidly smoother. Figure 12 shows the change that has taken place in such a union within a few months.

Detailed studies of the tissues along the line of union in an interspecific graft have indicated several anatomical features characteristic of this region. A particularly smooth union, several months old, was used in the studies illustrated below. It was formed by a long diagonal splice. The vascular elements had been set at a slight angle and the lack of alignment that persisted helped in differentiating the tissues of the two species. Figures 13–16 illustrate this union; they are so arranged that *N. glauca*, the stock, appears on the right and *N. tabacum*, the scion, on the left.

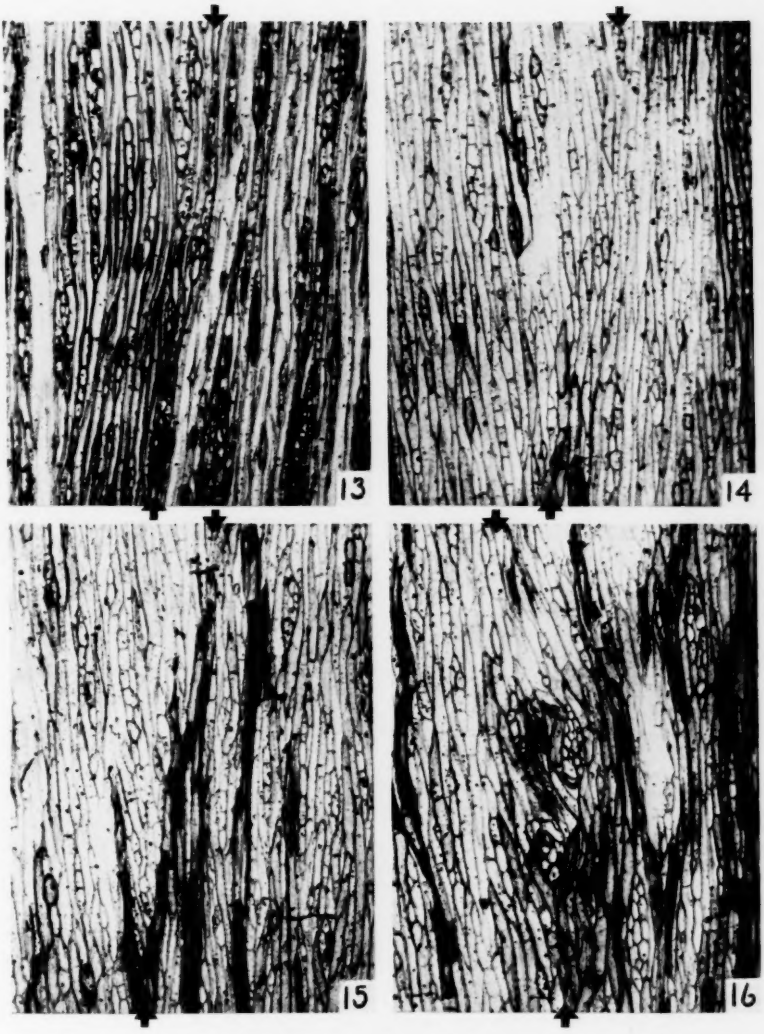
Figure 14 shows the union in the cambium; figure 15, in the phloem; and figure 13, in the xylem. The line of union is denoted by the arrows in the photographs. In the lower part of figure 15, opposite the arrow, a sieve tube may be seen to branch, one arm continuing on the *N. glauca* side and the other entering the *N. tabacum* tissue. Figure 17 is a more detailed illustration of the phloem along the line of union. Although the cells are somewhat misshapen, no irregularities in pitting could be found; and a special technique for illustrating plasmodesma (**5**) showed no abnormalities in these. The dark structure in the center is a lateral sieve plate, slightly out of focus.

In many unions studied, some abnormal formation of definitive callus occurred in sieve tubes of the stock. This was most pronounced when the line of union was transverse. Although definitive callus formation is characteristic of the old sieve tubes approaching obliteration in all stems, in this special case active sieve tubes right up to the cambium layer showed this condition, which was localized

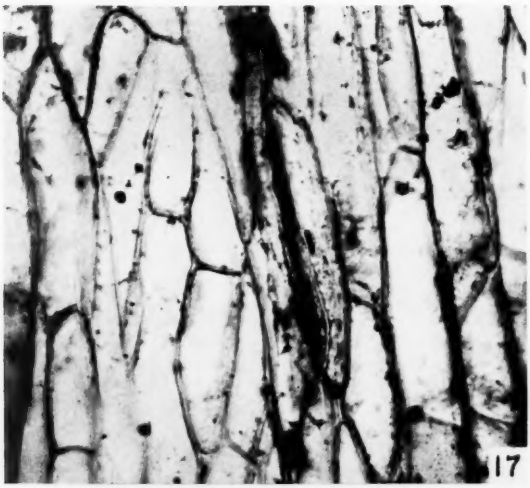




FIGS. 11, 12.—Fig. 11, secondary phloem in longitudinal tangential section from graft union. *N. glauca* on right, *N. tabacum* on left. ×45. Fig. 12, graft union of *N. glauca* (above) and *N. tabacum* (below). Secondary growth has proceeded for several months and radial distortion has been largely overcome. Paths of xylem vessels are still not straight in tangential plane as indicated by open vessel ends shown. Original cut surfaces of bark have been forced to exterior. Phloem elements lie in varied patterns, somewhat as in fig. 11, and sieve plates display excessive definitive callus. ×22.



FIGS. 13–16 (from sliding microtome sections of fresh material stained with iodine and anilin blue).—Fig. 13, longitudinal tangential section of xylem in region of graft union of *N. tabacum* on *N. glauca*. Fig. 14, similar section through cambium. Fig. 15, phloem of the union. Sieve tubes frequently branch. Fig. 16, phloem in a different location, showing "islands" of tissue commonly found along line of union. All ×83.



FIGS. 17–19.—Fig. 17, longitudinal tangential section of phloem on line of graft union. Figs. 18, 19, abnormal callusing of sieve plates of the stock, *N. glauca*, in interspecific graft; two views shown at slightly different foci. Characteristic sieve-tube plastids can readily be seen in fig. 18, even in heavily callused cells. ×430.

within a narrow band adjacent to the line of union. The plastids in these callused cells were full sized and prominent. In senile sieve tubes they decline and disappear. Figures 18 and 19 illustrate the callused sieve plates and numerous plastids in some of these sieve tubes.

In many unions there also occurred small groups or "islands" of cells along the line of junction. These could be found at the time of cambium initiation in new grafts and gave the appearance of root initials. Later adventitious roots were found on several scions, apparently being first formed in the meristematic tissues along the line of union. Figure 16 shows them in the older union previously described (figs. 13–16). Phloem parenchyma cells surrounding these "islands" were usually well filled with starch. The occurrence of root initials accompanied by food storage indicates that the channels of conduction in the phloem were partially clogged. There were no other signs of incompatibility, however, and the grafted plants were perfectly healthy.

Discussion

With the demonstration that the sieve tubes of two *Nicotiana* species behave like those of potato and the cucurbits, namely, that they become completely permeable with maturity, the assumption that this permeable condition is causally related to conduction in the phloem seems strengthened. BENNETT (**1**) has recorded rates of virus movement approximating those calculated for conduction of foods by the writer (**4, 6, 7**) and others (**9, 15, 16**). His ringing experiments with tobacco parallel those of physiologists in demonstrating the relation of phloem continuity to this movement. Other current studies (**11, 19**) indicate the essential part played by sieve tubes in conduction. Two further indications of the very specialized nature of secondary sieve tubes in tobacco are their multinucleate condition and their differentiation from callus parenchyma cells. Evidently these elements have unique properties peculiar to no other cells of the plant, during certain stages in their ontogeny are adapted to the function of conduction, and are the seat of several very sensitive responses. They should receive careful study by those interested in physiology as well as anatomy, both in healthy and in diseased plants.

The studies just described on the formation of the graft union bring out its relation to conduction. Evidently the scion is practically independent of the stock until the calluses intermingle. Then, in tobacco, vascular elements are soon differentiated and, from the physiological standpoint, the union is formed. Subsequent initiation of a cambium and establishment of normal secondary growth provide more efficient vascular connection and the supporting tissues necessary for prolonged existence of the new plant. Vascular continuity and provision for the required processes of conduction, however, seem to be made before the cambiums are united.

Certain writers (**3, 10, 18**) give the impression that, in woody species, continuity of the cambium is established first and that the earliest vascular elements are derived from this cambium. SASS describes the process as thus taking place in apple. Only when poor matching was followed by excessive callus formation and delayed cambial bridging did he find the oddly shaped xylem elements characteristic of the first xylem connections in tobacco. KOSTOFF does not mention the exact order of these processes in his grafts. Although he says, "The cambial connection appears first in the lowest region of the callus" (**13**, p. 570), he later describes and illustrates "abnormal vascular bundles" that correspond closely to the strands differentiated before cambium formation in the unions studied in the present work. BRADFORD and SITTOŃ (**2**) are somewhat indefinite on this particular detail. In discussing congenial unions (p. 47) they say, "Union occurs first in a zone of parenchymatous tissue, which becomes gradually cut up by tracheids, vessels, and fibers, and finally disappears. . . . . If the fit of stock and scion is particularly close, the zone is very limited in extent and quickly disappears." Here they seem to imply the formation of xylem strands within the callus parenchyma. In describing the union in the bark (p. 52), however, they state, "Sometimes . . . . there is a growth of parenchymatous nature from the cortical regions, establishing a union between stock and scion. Whether this is established or not, new bark is formed as soon as the cambium layers unite." Many of their illustrations show small oddly shaped xylem elements resembling those found in the tobacco graft unions. Continuity of the cambium possibly pre-

cedes differentiation of vascular elements in some species; however, since the vascular tissue that differentiates prior to cambium formation "quickly disappears," as just noted, it may have been overlooked by some investigators.

Regardless of the order in which these various tissues appear, vascular continuity in tobacco is rapidly established in the graft union. As soon as the elements become functional, paths are open for the movement of the curly top virus across the union. Since differentiation of xylem elements and sieve tubes occurs simultaneously, the finding of any mature vascular elements in a tissue bridge is good evidence that both these systems have become functional. There seems no reason to doubt that the ringed stems transmitting virus in BENNETT's studies (1) were bridged by wound callus within which functional phloem had differentiated. Apparently there are no exceptions to the theory that the transmission of curly top virus occurs only in the phloem.

Summary

1. The structure and development of sieve tubes seem causally related to normal conduction in plants, to the movement of certain viruses, and to the formation of the union in grafts and wounds.

2. Stages in the ontogeny of phloem elements in tobacco differ little from those described in potato. Primary sieve tubes contain slime bodies, nuclei, plastids, and the usual cytoplasmic structures. The young elements accumulate neutral red and may be readily plasmolyzed. As they mature, the nuclei and slime bodies disintegrate, vital stains do not accumulate, and the cells can no longer be plasmolyzed.

3. Secondary sieve tubes have no slime bodies. As they leave the cambium, the nuclei may divide one or more times to form as many as four nuclei in the differentiating cell. Later these nuclei disintegrate, plastids develop, and the cells acquire the usual properties of mature sieve-tube elements. Obliteration follows the same course as in potato.

4. Vascular strands differentiated from callus parenchyma connect stock and scion within as few as five days after grafting.

5. Cambium arises within these strands; and by lateral extension a complete layer is soon formed, continuous with the cambiums of the stock and scion.

6. Orientation of the cambium initials depends upon that of the original vascular strands. Where the matching of stock and scion is good, the strands are short and straight, and the cambium approaches in form that of the normal stem. Where matching is poor, the strands are long and crooked, while the cambium initials lie in varied patterns. Grafts of this type are so rough that conduction may be hindered. When no incompatibility exists, they usually become smoother with time.

7. Formation of definitive callus occurs abnormally early along the line of union, especially on the side of the stock. Sieve-tube plastids in these callused cells are normal in form and numbers.

8. No irregularities in pitting of cell walls could be found along the line of union in the *N. glauca-N. tabacum* grafts.

9. Root initials, surrounded by parenchyma cells heavily stored with starch, characterized the line of union in several grafts.

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EXPLANATION OF PLATES XI, XII

PLATE XI

FIG. 1. Young sieve-tube elements of *N. tabacum*, showing slime bodies, nuclei, and vacuoles in cytoplasm. ×455.

FIG. 2. Slightly older sieve-tube element with slime body somewhat fragmented and nucleus expanding prior to disintegration. A mature element appears on left. ×455.

FIG. 3. Sieve plate of *N. tabacum* with slime plug composed of fragments of disintegrated nucleus and slime body. ×455.

FIG. 4. A more mature element showing amorphous disintegration products; plastids conspicuous in last three views. ×455.

FIG. 5. Young sieve-tube element of *N. glauca*, slightly more mature stage than in fig. 1. ×455.

FIG. 6. Fragmenting slime body in *N. glauca*. ×455.

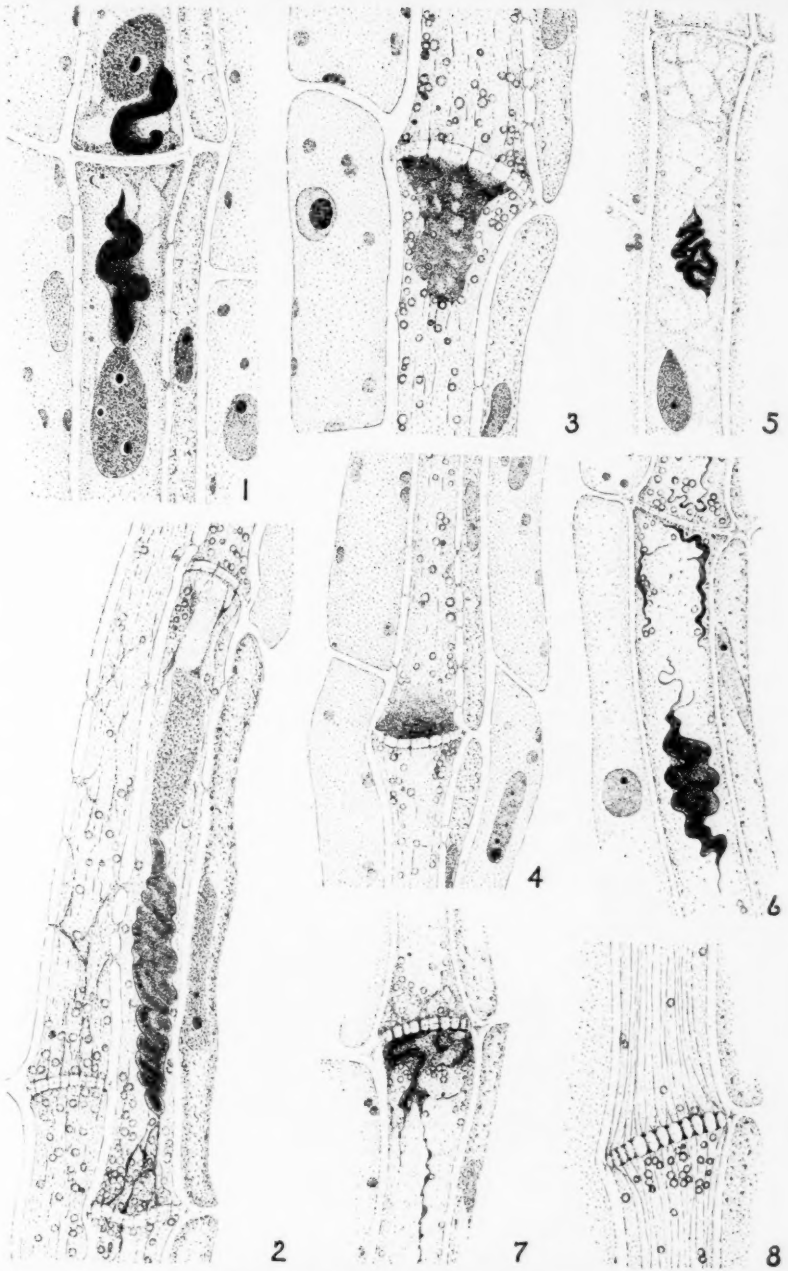
FIG. 7. Sieve plate of *N. glauca* with slime plug composed of distinct fragments of the slime body. ×455.

FIG. 8. Sieve plate of mature element in *N. glauca* in which all disintegration products have left the cells, while the protoplasm shows clearly a parietal layer and an internal stranded condition. ×455.

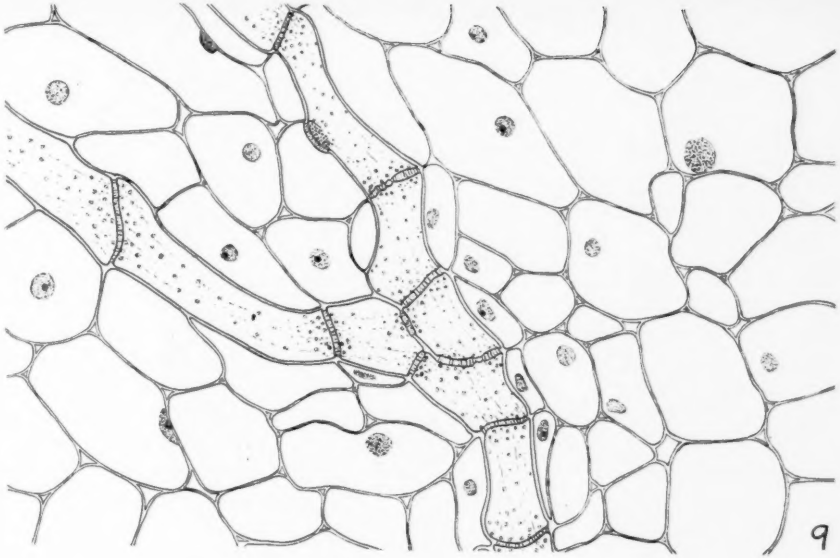
PLATE XII

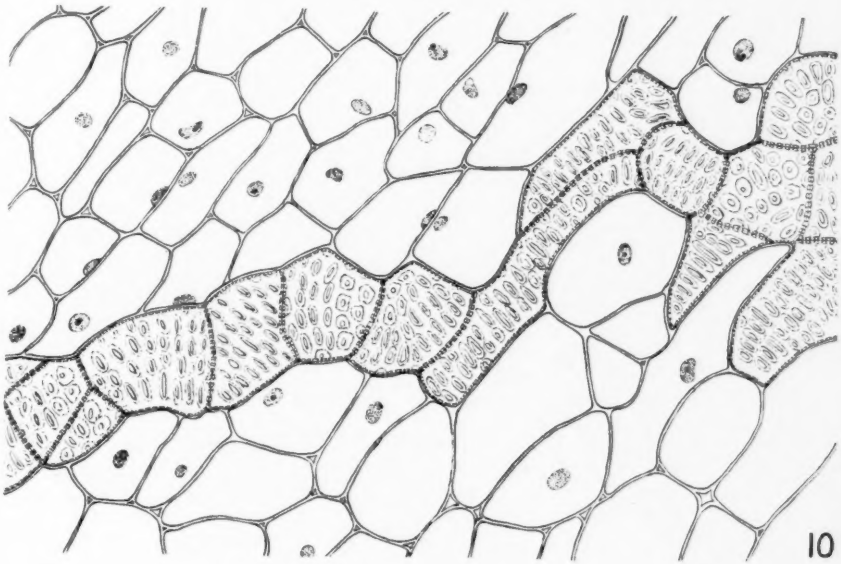
FIG. 9. Sieve tube differentiated from callus parenchyma cells within callus tissue of graft. ×410.

FIG. 10. Xylem vessel formed by differentiation of callus parenchyma cells. Shape, size, and matching of these elements indicate their origin from callus cells. Walls between xylem elements contained large pores; true vessels are evidently formed by this process. ×410.



CRAFTS on PHLOEM ANATOMY





CRAFTS on PHLOEM ANATOMY

A CYTOLOGICAL ANALYSIS OF SELF-STERILITY IN TRADESCANTIA

EDGAR ANDERSON AND KARL SAX

(WITH THREE FIGURES)

Introduction

Many species of *Tradescantia* are strongly self-sterile. MOORE (**6**) reported 83 self-pollinations as completely sterile, and in all the cultures of *Tradescantia* now under cultivation at this institution no self-fertile plants have as yet been discovered. Table I summarizes

TABLE I

SPECIES	NAME AND NO. OF PLANT	NO. OF SELF-POLLINATIONS	NO. OF SWOLLEN CAPSULES	NO. OF SEEDS RIPENED
T. reflexa	Algonquin 52	25	0	0
T. reflexa	Ullin 20	25	0	0
T. reflexa	Dexter 1	25	0	0
T. gigantea	Gigantea 4T	8	0	0
T. montana	Balsam Gap 10	25	0	0
T. bracteata	Portage des Sioux 47	10	3	0

the results of a series of controlled self-pollinations. In only one plant was there any indication of self-fertility, although all the plants used in the experiment had been proved fertile in crosses with other individuals, averaging a little more than three morphologically perfect seeds per pollination. Even in this one instance the plant was not truly self-fertile, since the few seeds which developed were small, shrunken, and practically empty. A cytological analysis of self-sterility was therefore undertaken, since the large nuclei and clear cytoplasm of *Tradescantia* facilitate investigation which in other genera would be difficult, if not impossible.

Investigation

METHODS.—Practically the entire study was based upon aceto-carmine smears of self and cross-pollinated styles. The plants used were grown in pots in the experimental greenhouse. Before *Trades-*

cantia flowers open they are sensitive to handling and bud emasculation is not ordinarily practicable. The flowers open shortly before sunrise (varying somewhat with the temperature and the humidity), and pollen is not ordinarily shed until much later in the morning, particularly in the case of plants kept in the greenhouse. It is therefore possible to emasculate the plants satisfactorily after the flowers have opened. Pollen was used from freshly opened flowers, and as far as possible the work was carried on during sunny weather. It has been found (unpublished data) in *Tradescantia* that during cool cloudy weather the percentage of imperfect pollen is greatly increased. A few of the pollinations were made on plants kept for a day or two in the laboratory, but for the most part plants were pollinated in the greenhouse and the styles brought into the laboratory. When the length of the pollen tube was in doubt the whole style was smeared, but when it was approximately known, the "empty" lower portion of the style was cut away before the smear was made. In a few cases the styles were slit in half with a dissecting needle before they were mounted. The styles were placed on a slide, covered with a drop of aceto-carmine and a cover glass, and then pressed gently but firmly until they were smeared flat. It was found advantageous to heat the smears gently until they were almost up to the boiling point; the preparations were then sealed with wax and were ready for use. The lengths of the pollen tubes were obtained by making camera lucida drawings at bench level and measuring the drawings.

As seen in aceto-carmine smears, the ungerminated pollen grain of *Tradescantia* is comparatively homogeneous, with a dark-staining, sharply defined generative nucleus and a much less well-defined vegetative nucleus (**8**). The generative nucleus is already a coiled body, many times longer than broad, and becoming progressively longer as germination proceeds. It assumes various positions, although it most characteristically assumes a coiled and twisted U-shape. It is often possible to show that the two ends of the U are unlike, since one is terminated by a short and very definite knob or granule (fig. 1 *C*). In many preparations the nucleus has the appearance of being divided longitudinally by a deep crease, and sometimes an actual fissure is visible nearly the whole length of the nucleus.

The vegetative nucleus is much more hazy and amorphous. There is some evidence of organization and very often an appearance of more or less parallel lines of a deeply staining material. The two nuclei are characteristically closely associated. They may be at either end of the grain or in the middle, but except in rare cases they are either close together or are actually intertwined. The commonest position of the vegetative nucleus is between the arms of the generative nucleus, as shown in figure 1. Germination may take place at one end of the pollen grain, at both ends, or along the side, the first condition being by far the most common. The grain apparently germinates at the point where it touches the stigma. Since the grains are longer than broad they naturally are lodged endwise, among the long papillae of the stigma (fig. 2), and germinate from the proximal end. In the one or two instances where grains were observed to have germinated along the side, it could be seen that they were lying with their long side against the stigmatic papillae. Neither nucleus leaves the grain until the tube has reached a considerable length. Although the vegetative nucleus usually precedes the generative nucleus down the tube, it does not invariably do so. Numerous cases were found in which the generative nucleus was well in advance.

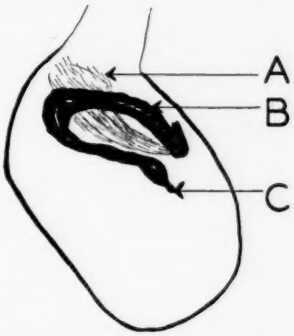


FIG. 1.—Germinating pollen grain, drawn with camera lucida (*A*, vegetative nucleus; *B*, generative nucleus; *C*, granule at one end of nucleus). ×900.

Compatible and incompatible pollinations were made simultaneously on different flowers of the same plant, and were examined at intervals of from 15 minutes to 3 days. The difference between the two types of mating was apparent as soon as 15 minutes after pollination and became increasingly conspicuous thereafter. Germination apparently took place almost immediately in both cases, but the incompatible matings were characterized by slow growing pollen tubes. In the compatible matings, the generative nuclei began to leave the grains and pass down the tubes in about 40 minutes to an hour

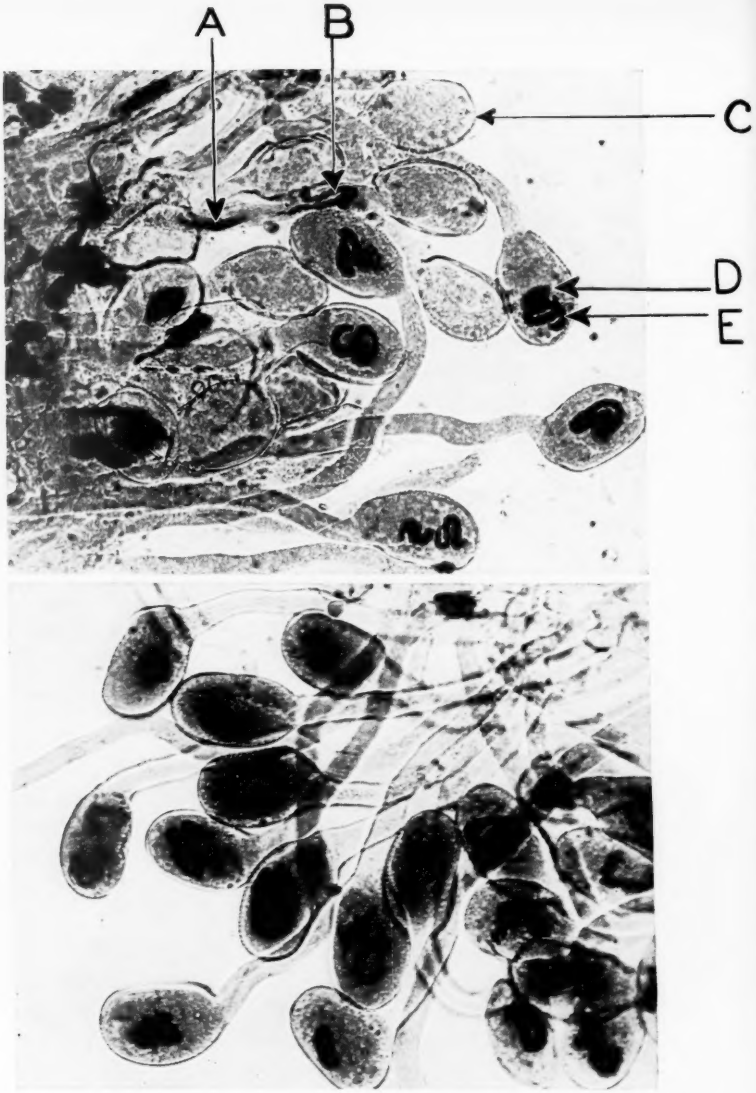


FIG. 2.—Photographs of aceto-carmine smears of pollinated *Tradescantia* styles. Above: cross-fertile (compatible) pollination; below: self-pollination (incompatible). Smears made 5 hours after pollinating (*A*, vegetative nucleus passing down pollen tube; *B*, generative nucleus in same tube; *C*, pollen grain from which nuclei have departed; *D*, vegetative nucleus in pollen grain; *E*, generative nucleus in same grain). Note denser cytoplasm and absence of "empty" pollen grains in the incompatible mating.

while in the case of incompatible pollinations few of the nuclei left the grain within the first 24 hours. The records of a typical experiment are given in detail in table II and photographs of smears are shown in figure 2. Comparative growth curves for compatible and incompatible matings are shown in figure 3. It is difficult to construct really accurate growth curves for two reasons. In the first

TABLE II

COMPARISON OF SMEARS FROM CROSS-FERTILE AND CROSS-STERILE MATINGS; POLLINATIONS MADE IN THE LABORATORY ON POTTED PLANTS OF T. GIGANTEA

TIME	CROSS-FERTILE	CROSS-STERILE
30 min.	7×3 Three-quarters of grains germinated; tubes mostly longer than grain; longest tubes six times length of grain; generative nuclei all in grains	7×2 Only a few grains germinated; tubes mostly shorter than grain; longest tubes scarcely longer than grain; generative nuclei all in grains
1 hour	2×3 Nearly all grains germinated; nuclei mostly in grains, a few in tubes	2×7 A few grains germinated; longest tube four times length of grain; nuclei all in grains
2 hours	2×3 Many nuclei in tubes; several tubes in lower half of style	2×7 Nuclei all in grains; no tubes in style proper
8 hours	7×3 Many tubes half-way down style, a few at base; over half of nuclei in tubes	7×2 No tubes half-way down style, longest barely beyond stigma; nuclei all in grains

place different pollinations on the same plant, made within one or two minutes of each other, may vary 15 minutes in the onset of germination. In the second place there is such great variation between the individual pollen tubes in any compatible cross that it is difficult to arrive at a reliable average. The longer tubes are much more likely to break in the process of making the smear than the shorter ones. Figure 3 shows the range in variation of tube length as well as the average length, but it should be remembered that for the two-hour period the latter figure, in the case of the compatible mating, is little more than an estimate.

One point, however, is clear. The growth curve for the incom-

patible matings is depressed; there is some inhibitory process at work which is progressively slowing down the rate of pollen tube growth. It is apparent within the first few minutes of growth and becomes increasingly conspicuous as growth proceeds. Whether, on the other hand, the compatible tubes grow at a constant or at an accelerating speed cannot be determined from the available data. In the com-

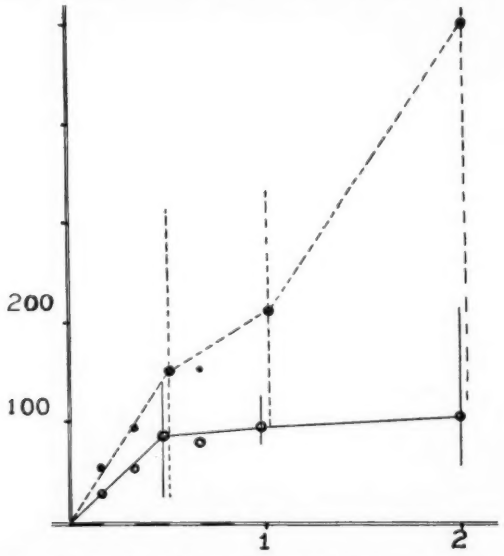


FIG. 3.—Growth of pollen tubes in compatible (broken line) and incompatible (solid line) crosses. Below: time in hours; vertical scale: pollen tube length in 100 μ. The lines connect averages (medians) and the vertical broken lines extend to the extreme values obtained at each interval. The six unconnected points at 10, 20, and 40 minutes are the averages obtained in another set of experiments.

patible crosses pollen tubes were found at the base of the style in four to five hours. The longest tubes found in incompatible crosses of this age were only 150 μ. Since the styles of *Tradescantia* were about 6–7 mm. long, the growth rate of the compatible crosses was about 25 μ per minute and that of the incompatible one about 0.5 μ per minute.

In the case of the incompatible matings the uniform color of the cytoplasm could not be observed to change in smears made at suc-

cessive intervals after pollination. In the compatible matings it became increasingly differentiated (fig. 2) and large vacuoles were often visible. The ends of the pollen tubes assumed different shapes in different experiments. They were sometimes slightly swollen, sometimes of the same diameter as the tube itself, and sometimes pointed. On the whole there seemed to be an association between pointed ends and incompatible matings and between swollen ends and compatible matings, although apparent exceptions were noted. The whole matter, like many others connected with these experiments, is difficult of determination because of the extreme variability which seems to characterize pollen tube behavior.

In many self-sterile species, pollination tests have revealed the presence of cross-sterility between different individuals. In many of these genera it has been possible to group the individuals into a number of intra-sterile, inter-fertile classes, all the members of each class behaving, so far as their compatibilities are concerned, like a single self-sterile individual. (See WILLIAMS and SILOW **11**, p. 344, for a summary of genera and review of literature.) An attempt was accordingly made to see whether it might be possible to diagnose cross-sterility by purely cytological methods. Crosses made at random among *Tradescantia* plants collected from the same locality revealed the presence of many cases of cross-sterility. Cytologically no qualitative nor quantitative difference could be found between these cross-sterile matings and self-pollinations. The rate of pollen tube growth was the same, and the nuclei had the same dark turgid appearance.

In the diploid species and varieties, compatible and incompatible matings were so strikingly different when examined cytologically that it was possible to work out the composition of the intra-sterile, inter-fertile classes by cytological examination alone. As a check on the cytological ratings, a number of matings, recorded as cross-sterile or cross-fertile by cytological examination, were actually made as controlled pollinations. In every case there was complete agreement between the cytological prediction and the actual compatibilities.

Three diploid species were studied intensively, *T. gigantea* Rose, *T. humilis* Rose, and *T. edwardsiana* Tharp. All three gave the same

general results, although they differed in minor points. The division into classes was sharpest in *T. gigantea* (table III), that is, there were no plants whose class relationships were in doubt. In *T. humilis* there was a single exceptional plant (no. 3, table III) which showed an intermediate degree of cross-fertility with all the members of one class, both when used as a male and as a female. In *T. edwardsiana* there was some indication of one intra-sterile class, but

TABLE III

COMPATIBILITIES BETWEEN PLANTS OF SINGLE LOCALITY IN T. GIGANTEA AND T. HUMILIS: −, INCOMPATIBLE MATING; +, COMPATIBLE MATING

T. HUMILIS N.D.D.

♀ \ ♂	11	25	3	18	22	16	5	10	6
11	−	−	±	+	+	+	+	+	+
25	−	−	±	+	+	+	+	+	+
3	±	±	−	+	+	+	+	+	+
18	+	+	+	−	+	+	+	+	+
22	+	+	+	+	−	+	+	+	+
16	+	+	+	+	+	−	+	+	+
5	+	+	+	+	+	+	−	+	+
10	+	+	+	+	+	+	+	−	+
6	+	+	+	+	+	+	+	+	−

T. GIGANTEA

♀ \ ♂	1	4	5	6	14	15	2	7	10	8	9	4u	4w	3
1	−	−	−				+			+			+	
4	−	−	−	−	−	−	+	+	+	+	+	+	+	+
5		−	−	−			+	+	+	+	+	+	+	+
6		−	−	−				+	+	+	+	+	+	+
14			−			−		+		+				
15														
2							−			+			+	
7		+	+	+	+	+	−	−	−	+	+	+	+	+
10			+	+				−	−	+	+	+	+	+
8	+	+	+	+			+	+	+	−	+	+	+	+
9		+	+	+				+	+	+	−	+	+	+
4u		+	+	+				+	+	+	+	−	+	+
4w	+	+	+	+				+	+	+	+	+	−	+
3		+	+	+	+	+	+	+	+	+	+	+	+	−

there were several inconsistencies. It was among these very plants of *T. edwardsiana*, however, that there were found the two individuals with interchanged chromosomes, producing mitotic irregularities which have been reported elsewhere (7). There is also a high percentage of interlocking between non-homologous bivalents in several of the plants of this collection. It is therefore possible that compatibility relationships are disturbed either directly or indirectly by chromosome interchange. If a considerable proportion of the plants in the colony were duplicate for the loci affecting self-sterility, one would expect the same lack of sharply marked classes which characterizes polyploid self-sterile plants (1). It does not seem profitable to discuss possible interpretations of the compatibility rela-

tionships in *T. edwardsiana* at greater length until there are available genetic as well as cytological data.

Very little is known about the number and distribution of intra-sterile, inter-fertile classes in natural populations. EAST and his co-workers (**2**, see detailed bibliography) have reported the presence of a great number of such classes in experimental populations of *Nicotiana*. In *Antirrhinum*, GRUBER (**5**) has reported that the number of such classes distributed in nature must be very great. In *Tradescantia* there are apparently an enormous number of such classes in natural populations. In *T. gigantea* seven classes were identified among the 14 plants collected and studied. These plants came from two small areas, situated no more than 300 feet apart, and neither one larger than a table top. In *T. humilis* 9 classes were found among the 12 plants collected in a single field. In *T. edwardsiana*, among the 20 plants studied four belonged to one intra-sterile group. Aside from two other plants whose class relationships were uncertain, there were no other cases of cross-sterility.

Genetical interpretation

While in general the cytological data on self-sterility in *Tradescantia* are in accord with what we might expect on the oppositional factor hypothesis, there are several points of minor disagreement, as will be noted below. It may therefore be profitable, before discussing the genetical interpretation of the results, to consider what kinds of relationships between the pollen tube and the style might possibly obtain in self-sterile plants. The preceding cytological data give clear evidence on several points and effectively rule out certain possibilities. Pollen germination is not affected. The reaction between the pollen tube and the style is apparent almost immediately after germination and seems to be general throughout the style. This agrees with the condition reported for *Nicotiana* (**4**), but differs from the phenomena observed in *Trifolium* by SILOW (**9**) and in *Hemerocallis* by STOUT and CHANDLER (**10**). There is also clear evidence in *Tradescantia* that there is a strong inhibitory force at work in incompatible matings. On the basis of these findings one might possibly expect one or all of the following reactions.

1. (Oppositional factor hypothesis). There might be a reaction be-

tween the tissues of the style and the factors carried by the pollen. Such a reaction would account for most of the phenomena of cross- and self-sterility in *Nicotiana* (3) and a number of other genera. With simple extensions the same hypothesis can be applied successfully to polyploids (1).

2. There might be a reaction between the tissues of the style and the pollen tube, determined not by the factors carried by the pollen itself but by those of the plant on which it was borne. One might expect such a reaction to be much more likely between the pollen grain and the stigma, than between the pollen tube and the style.

3. The reaction of either (1) or (2) might influence the germination and growth of other pollen.

According to the oppositional factor hypothesis, the operation of self- and cross-sterility (in a diploid) would be explained as follows: Pollen grains carrying genetic factors identical with those of the style would be inhibited, and although they might germinate would not grow fast enough to cause fertilization. Plants of identical constitution for these essential factors would be completely cross-sterile and plants having one factor in common would be semi-compatible, although this partial sterility might be apparent only by genetical tests. For example, if a plant of the constitution S_1S_2 were crossed with a plant of the constitution S_1S_3, half of the pollen would carry S_1 and half S_3. The former would be inhibited by the action of the S_1 factor in the style and the resulting progeny would be of only two classes, S_1S_3 and S_2S_3.

The *Tradescantia* plants available for cytological examination consisted of a number of samples of natural populations. If the oppositional factor hypothesis as just outlined is responsible for the incompatibilities in these populations, there are a number of means by which it should be possible to test the matter cytologically, as follows:

1. There should be inter-fertile, intra-sterile classes. These were found as has been reported.

2. There should be two grades of cross-fertile matings, the fully compatible (type $S_1\ S_2 \times S_3\ S_4$) and the semi-compatible (type $S_1\ S_2 \times S_1\ S_3$) explained above. On this point the results are not conclusive. The smear technique employed does not make it possible to follow

the majority of pollen tubes after they have left the upper portion of the style. Compatibility may be measured in a rough manner by determining the percentage of pollen grains which germinate but in which the nucleus does not leave the grain promptly. Counts were made on styles smeared 8 hours after pollination for 30 different compatible matings in *T. gigantea*. The percentage of germinated grains whose nuclei had gone down the tube ranged from 24 to 76 per cent. The percentage formed a unimodal distribution, with over half of them falling between 40 and 60 per cent. This may possibly mean that all of the matings tested were semi-compatible with an expecta-

TABLE IV

LENGTHS OF POLLEN TUBES, IN μ, 30 MINUTES AFTER POLLINATION. FROM SMEARS OF SELF-STERILE AND CROSS-FERTILE POLLINATIONS IN TRADESCANTIA GIGANTEA

LENGTH OF POLLEN TUBES (μ)													
0–24	25–	50–	75–	100–	125–	150–	175–	200–	225–	250–	275–	300–325	
2	1	14	9	2	2	....	....	....	....	....	....	....	plant no. 4W selfed
1	0	1	0	5	4	5	0	2	0	1	0	2	plant no. 5 × no. 4W

tion of 50 per cent, or it may mean that the oppositional factor hypothesis in its simplest form cannot be applied to *Tradescantia*.

3. In a semi-compatible mating there should be two main groups of pollen, the inhibited and the uninhibited, and they should occur in approximately equal numbers. Measurement of all the pollen tubes should show a bimodal distribution of tube length. The best data obtainable by the smear technique described are presented in table IV. It will be seen that there is no evidence for a bimodal ranking of the pollen tube lengths. This may possibly mean that the oppositional factor hypothesis will not account for the phenomena of cross-sterility in *Tradescantia*. On the other hand, the expected bimodality may possibly be obscured by modifying factors or by an interaction between the compatible and the incompatible pollen tubes.

In the absence of any genetical evidence, it does not seem profitable to discuss these and other possibilities at greater length. Briefly the cytological evidence is in line with the main requirements of the oppositional factor hypothesis, although it does not demonstrate the sharp difference between semi-compatible and fully compatible matings which would be expected on the simplest interpretation of that hypothesis.

Summary

1. Germinating pollen grains of *Tradescantia* are described as they appear in aceto-carmine smears of pollinated styles.

2. Many species of *Tradescantia* are self-sterile.

3. Compatible and incompatible pollination can be distinguished cytologically. The pollen grains germinate normally in the latter, but their growth is inhibited.

4. Cross-sterile pollinations cannot be distinguished quantitatively nor qualitatively from self-sterile pollinations.

5. Natural populations of diploid *Tradescantia* can be divided into intra-sterile, inter-fertile classes on the basis of pollen tube behavior.

6. The number of these classes in natural populations is found to be large.

7. The main facts revealed by this purely cytological examination are in line with the oppositional factor hypothesis although there is disagreement on a few minor points.

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ONTOGENY OF THE PRIMARY AXIS OF SOJA MAX

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 452

WILLIS H. BELL

(WITH THIRTY-SEVEN FIGURES)

Introduction

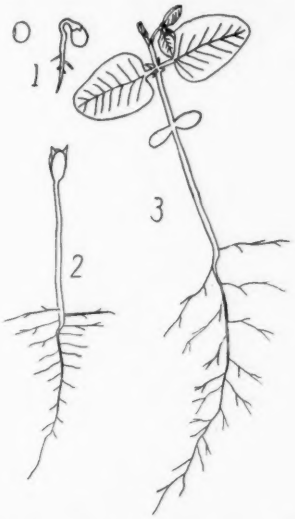
A voluminous literature dealing with nodule development and physiological relationships of the soy bean (*Soja max* Piper) has accumulated, but apparently the only strictly anatomical work has been a paper on the floral development by GUARD (4) and a brief seedling investigation by COMPTON (2). The present study has been undertaken to investigate the development of the primary axis. The variety used was the commercial Mammoth Yellow. PIPER and MORSE (6) have given the following description of this variety: "Plants stout, erect, bushy, maturing in about 145 days, pubescence gray; flowers white." At maturity the plants may be either bushy, with only a few internodes elongated, or vine-like, with more internodes elongated, depending upon physiological conditions of growth. The first leaves are simple and opposite and at right angles to the plane of the cotyledons. All other leaves are alternate and trifoliately compound. The flowers of this variety are in axillary racemes. The aerial portions of the plant are pubescent with simple hairs.

Investigation

The plants were grown under greenhouse conditions, and seedlings ranging from two to twenty days old were fixed for study. The stem and hypocotyl were fixed either in formal-acetic-alcohol or in chromo-acetic acid, and the root in chromo-acetic acid. The material was run up through alcohol and chloroform, imbedded in paraffin, sectioned at 12 μ, mounted serially, and stained with safranin and gentian violet. Drawings were made with the aid of a micro-projector and camera lucida.

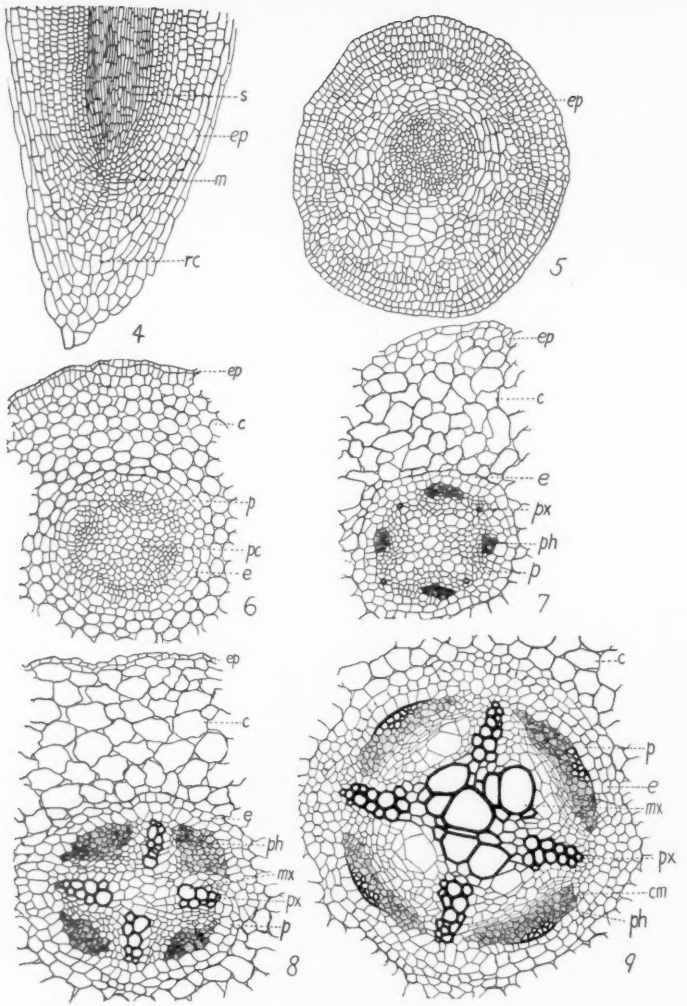
GERMINATION.—After imbibition of water the young primary root pushes through the seed coats and rapidly establishes itself as

an absorbing organ (fig. 1). The hypocotyl elongates, pulls the cotyledons up through the soil, and the seed coats are shed before the cotyledons appear at the surface of the ground (fig. 2). The cotyledons remain folded together as in *Phaseolus* until they are several centimeters above the surface of the soil. The epicotyl elongates but little until after the cotyledons unfold, and then it develops rapidly (fig. 3).



FIGS. 1–3.—Fig. 1, early stage in germination. Fig. 2, stage in germination after final elongation of hypocotyledonary arch. Fig. 3, young seedling with first true leaves fully developed.

PRIMARY ROOT.—The primary root at the completion of primary differentiation is a tetrarch radial protostele (fig. 9). It is differentiated from a meristematic group of cells at the tip of the hypocotyl (fig. 4). Development is according to JANCZEWSKI's (5) "fourth angiospermous type," in which the stele, cortex, epidermis, and root cap do not arise from definite histogens but from a common group of meristematic cells (fig. 4). The root cap is the first tissue differentiated, and consists of several layers of loosely packed parenchyma cells overlying the root tip. The epidermis is uniseriate and develops



FIGS. 4–9.—Fig. 4, longitudinal section of root tip (*s*, stele; *ep*, epidermis; *m*, meristem; *rc*, root cap). Fig. 5, cross-section of primary root above root cap showing epidermis differentiated. Fig. 6, same at time of differentiation of stele and cortex (*c*, cortex; *p*, pericycle; *e*, endodermis; *pc*, procambium). Fig. 7, same showing first protoxylem and protophloem (*ph*). Fig. 8, same showing primary xylem partially differentiated (*px*, protoxylem; *mx*, metaxylem; *ph*, primary phloem). Fig. 9, same after cambial activity has been initiated (*cm*, cambium).

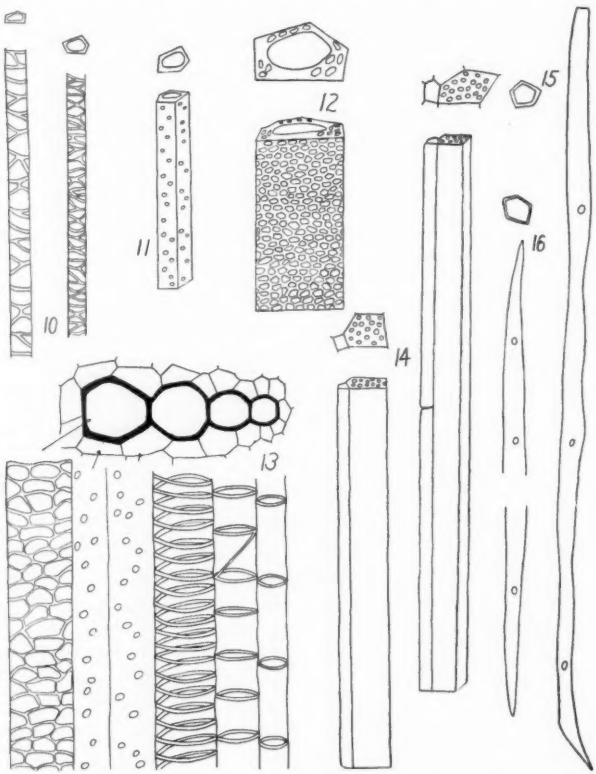
root hairs early in ontogeny (fig. 5). The cells of the cortex, except those of the endodermis, become large and loosely arranged with intercellular spaces while the cells of the stele are smaller and more compact (fig. 6). The primary xylem is exarch in its development, the first cells of the protoxylem differentiating from the procambium at four points adjacent to the pericycle (fig. 7). The one to three protoxylem vessels differentiated at each of the four points show spiral-reticulate markings on their walls (fig. 10). Metaxylem is matured progressively inward from the protoxylem points (fig. 8). The first metaxylem vessel segments have pitted walls with a single row of pits in each (fig. 11). The undifferentiated cells at the center of the primary root enlarge considerably and differentiate as metaxylem (fig. 9). The majority of these cells develop into short vessel segments with densely pitted walls (fig. 12). Some of the cells of this later developing metaxylem remain parenchymatous and ultimately differentiate into thick walled connective tissue.

The cells lying between the protoxylem points and adjacent to the pericycle differentiate centripetally as primary phloem (fig. 7). The first cells of the primary phloem remain parenchymatous and are crushed against the pericycle upon the initiation of cambial activity (fig. 9). The cells immediately beneath this parenchyma develop into phloem fibers. The remainder of the primary phloem consists of scattered parenchyma cells, sieve tubes, and companion cells. The sieve tube may be the same length as the companion cell or it may be equivalent in length to two companion cells (fig. 14). Cambial activity is initiated while the cells at the center of the root are still parenchymatous. At first activity is greatest immediately beneath the phloem, resulting in the crushing of the protophloem (fig. 9).

At about this time or somewhat earlier, the pericycle gives rise to secondary roots which dissolve their way through the cortex as they develop. The development of the primary body of the secondary root is identical with that of the primary root, except that ordinarily only one large metaxylem vessel instead of several is matured in the center of the root. The secondary root is usually smaller than the primary root, and may be diarch, triarch, or tetrarch.

Hypocotyl.—The hypocotyl as interpreted in this paper is that portion of the plant axis between the cotyledons and the true root.

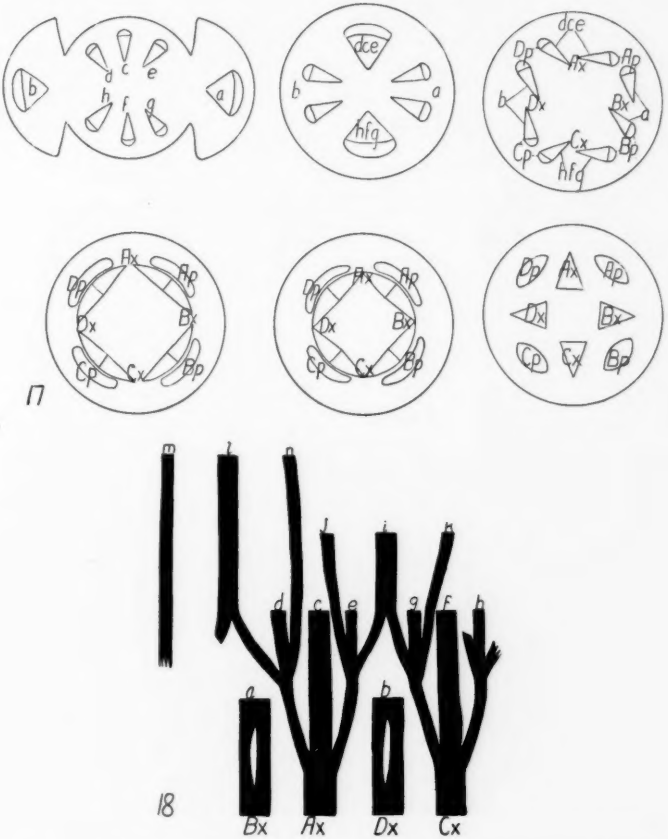
The lower portion of the hypocotyl is rootlike while the upper part is similar to the stem in the orientation of its tissues (figs. 19, 26). Upon germination the hypocotyl elongates as a result of meristematic



FIGS. 10–16.—Fig. 10, histology of protoxylem vessels of root. Fig. 11, histology of first metaxylem vessels differentiated in root. Fig. 12, histology of metaxylem vessel in center of root. Fig. 13, histology of primary xylem of stem. Fig. 14, sieve tubes and companion cells from root. Fig. 15, histology of phloem fibers of root. Fig. 16, immature pericyclic fiber from stem.

activity in the region of the hypocotyledonary arch (fig. 1). Procambial differentiation in the embryonic and elongating hypocotyl results in continuity of the procambium of the root and of the double

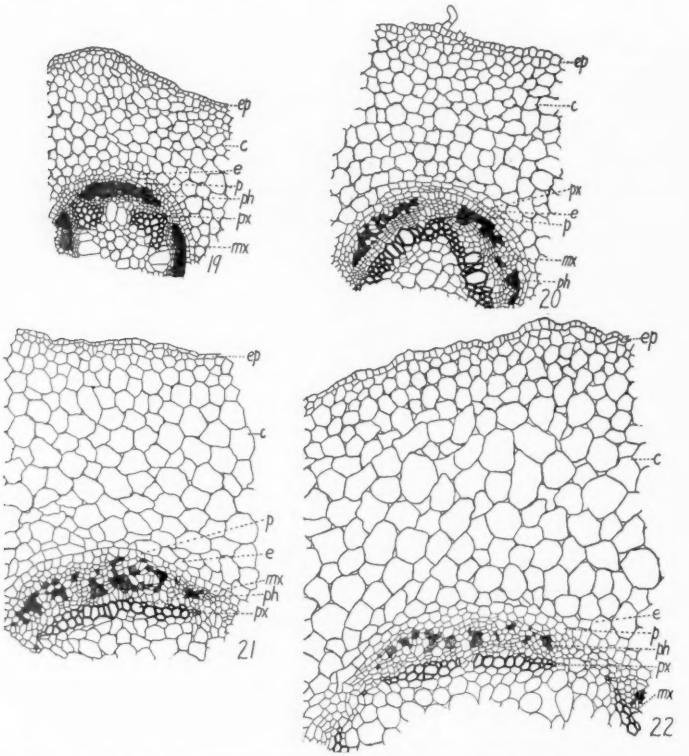
bundle to each cotyledon. Reorientation of the procambial strands in the lower part of the hypocotyledonary axis results in a transition



FIGS. 17, 18.—Fig. 17, diagrammatic cross-sections at different levels of the hypocotyl, illustrating method of root-stem transition and relationship of vascular supply of cotyledons and epicotyl to that of root. Fig. 18, diagram of main vascular supply of cotyledons and leaves at first three nodes.

between the radial protostelic structure below and the endarch collateral condition above. In this manner the four endarch collateral

bundles representing the traces to both cotyledons are differentiated against the tetrarch radial protostele of the root (figs. 17–26).



FIGS. 19–22.—Fig. 19, cross-section of hypocotyl just above level of root showing xylem groups about to bifurcate. Fig. 20, same showing siphonostele as a result of xylem divergence in root-stem transition. Fig. 21, same showing xylem divergences in contact by their metaxylem. Fig. 22, same showing xylem divergences entirely separated by parenchyma.

The exact manner of reorientation of the hypocotyledonary vascular system is shown in figures 17 and 18. The double bundle *a* of one cotyledon passes downward as two groups, which upon undergoing transition just above the root tie against the xylem *bx* and one wing of the phloem *bp* and *ap* of the root. The double bundle *b* to the

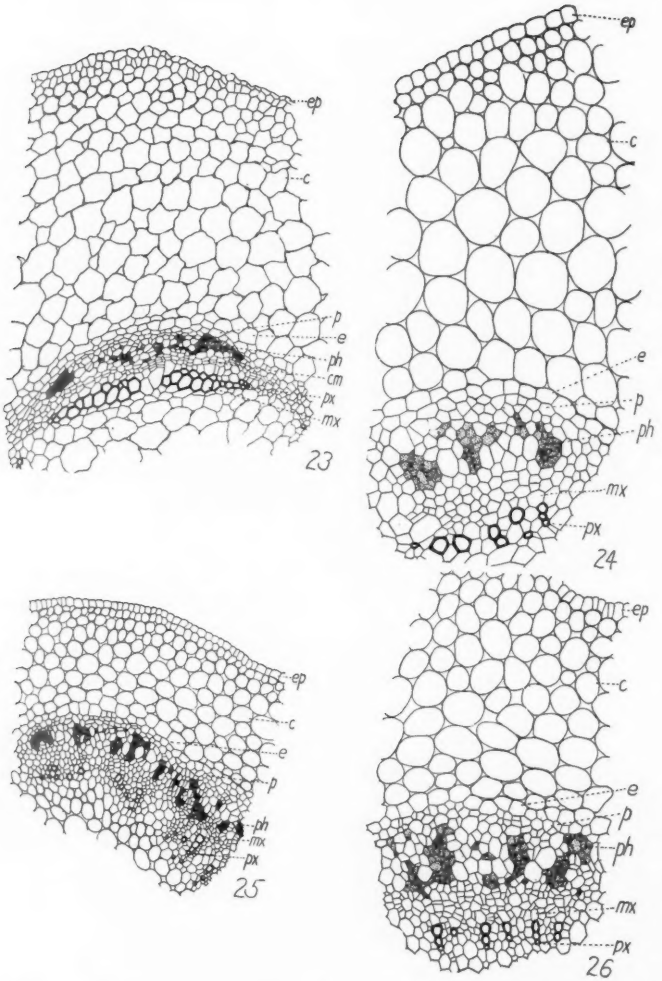
other cotyledon is differentiated against xylem *dx* and one wing of the phloem *cp* and *dp* of the root.

COTYLEDONS.—The cotyledons are sessile and at maturity are fleshy and oval in shape (figs. 3, 29). The veins of the cotyledons are divergences from the double bundle entering at the base. After maturation of the opposite leaves the cotyledons are dropped from the plant.

EPICOTYL.—Prior to germination the epicotyl of the embryo has differentiated several foliage leaves. The first leaves, which are simple and opposite, are differentiated at right angles to the plane of the cotyledons. The leaf primordium arises as a conical crescentic divergence from the apical meristem, which because of meristematic activity broadens laterally at its base until the entire periphery of the growing point is involved. This lateral activity results in the differentiation of two stipules for each leaf. The leaf primordium elongates with the cells at its apex differentiating a blade and the cells at the base elongating to form the petiole (fig. 27).

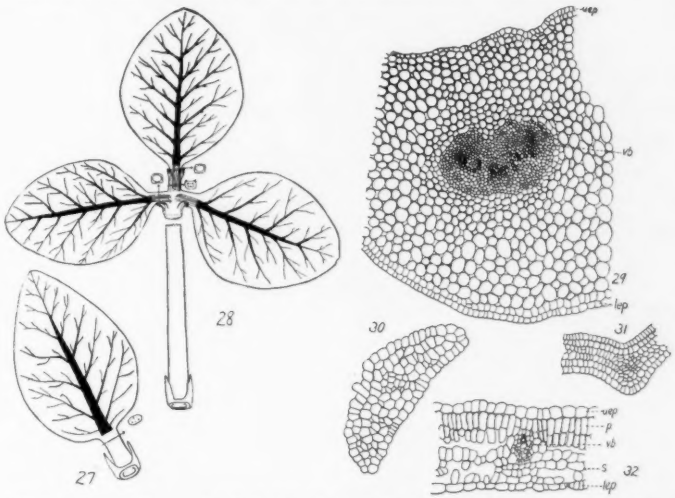
The primordia of the axillary flower buds are laid down at about the same time as are the associated leaf primordia. The procambial strands of the axillary bud are anastomosed with the downwardly differentiating strands of higher leaves at the node immediately below the point of divergence of the axillary bud.

In the development of the vascular system of the leaf the lateral bundles to the stipules converge with the median bundle at the base of the petiole. Meristematic activity continues in each stipule, resulting in the differentiation of a mesophyll. The veins to the stipule are divergences from the lateral bundle. Above the point of convergence of the median and lateral bundles the vascular system of the petiole appears stemlike, with several bundles in annular arrangement. Near the base of the lamina these bundles converge again to form a crescentic vascular ring. The blade is differentiated from a group of meristematic cells at the apical end of the leaf primordium (fig. 30). At first meristematic activity is general throughout, resulting in six to eight layers of cells (fig. 31). Differential growth eventually develops a blade with a single palisade layer and typical spongy parenchyma (fig. 32). Stomata are present in both the upper and lower epidermis but are more abundant in the lower.



FIGS. 23–26.—Fig. 23, cross-section of hypocotyl through transitional level showing cambium differentiation. Fig. 24, same showing primary xylem and phloem of transitional stage broken up into groups prior to endarch collateral orientation. Fig. 25, same showing both endarch and transitional xylem. Fig. 26, same after endarch collateral situation has been entirely attained.

The procambial strands which ultimately form the vascular skeleton of the blade are outward extensions of the median and lateral bundles of the petiole. In the leaf primordium the median bundle matures first, with the lateral bundles differentiating and maturing slightly later. The downward extensions of both are progressively matured as the hypocotyl and first internode elongate.



FIGS. 27–32.—Fig. 27, habit sketch of simple leaf. Fig. 28, same of compound leaf. Fig. 29, cross-section of a cotyledon (*uep*, upper epidermis; *vb*, median bundle; *lep*, lower epidermis). Fig. 30, cross-section of embryonic leaf. Fig. 31, cross-section of partially developed leaf prior to maturation of tissues. Fig. 32, cross-section of mature leaf (*p*, palisade parenchyma; *vb*, vascular bundle; *s*, spongy parenchyma).

One compound leaf is differentiated at right angles to the plane of the first pair of opposite simple leaves early in ontogeny (fig. 28). The development of this leaf is similar to that of the first true leaves just described. The median and lateral bundles of the trace are differentiated against the lateral bundles to the first leaves just above the cotyledonary plate. The stipules and petiole mature as just described but the petiole of the compound leaf undergoes much greater elongation. Instead of one meristematic group of cells at the tip of the primordium developing a blade, three regions of activity appear, giving rise to a trifoliate compound leaf, each leaflet receiving ap-

proximately one-third of the vascular supply of the petiole. Two small secondary stipules are developed as lateral divergences at the base of the terminal leaflet, and another pair immediately below the divergence of the two lateral leaflets (fig. 28). One small endarch bundle extends downward from each of these stipules and converges with the endarch groups to the leaflets which form the median and lateral bundles at the base of the petiole.

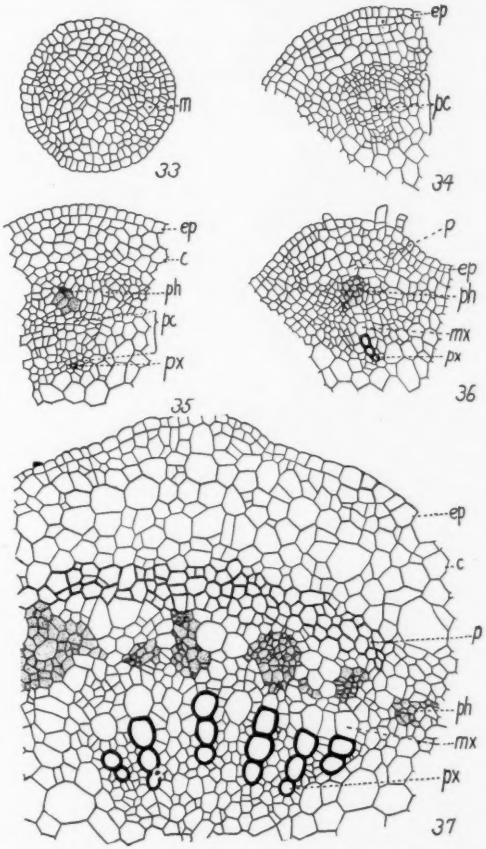
As seen in embryo, the median bundle *c* and the lateral bundles *d* and *e* of the trace to one of the opposite simple leaves pass downward and converge against the xylem *ax* and one wing of the phloem *ap* and *dp* of the root (figs. 17, 18). The trace *fgh* to the other opposite leaf converges with the xylem *cx* and one wing of the phloem *cp* and *bp* of the root. The trace *ijk* of the first alternate leaf is differentiated against the lateral bundles *e* and *g* of the opposite leaves at a level just above the cotyledonary plate (fig. 18). The trace *lmn* of the second alternate leaf converges against the lateral bundles *d* and *h* of the opposite leaves.

STEM.—The mature primary body consists of an endarch collateral dictyostele, pericycle, cortex, and epidermis (fig. 37). The pericyclic cells immediately external to the vascular bundles differentiate as fibers but in the region of the medullary rays remain parenchymatous. The primary vascular skeleton of the stem is a dictyostele consisting of the common bundles of the leaf traces.

The procambial strands may be recognized early by the greater density of the protoplasmic contents of their cells (fig. 34). The procambium adjacent to the undifferentiated parenchyma in the center of the stem matures as protoxylem (fig. 35). There are usually three strands of protoxylem (figs. 36, 37). The first strand shows widely separated annular markings (fig. 13) and is normally not collapsed at the time of maturity of the primary body. The later differentiated protoxylem strands show closer annular and spiral markings, the last formed consisting of tight spirals. Usually all the vessel segments have the histology of protoxylem but a few metaxylem vessels with pitted walls may develop. A large part of the primary xylem is parenchymatous at first and later matures as connective tissue.

Primary phloem is differentiated from the procambium in contact

with the pericycle and directly external to the primary xylem (figs. 35–37). The primary phloem consists of groups of sieve tubes and companion cells separated from each other by parenchyma (fig. 37).



FIGS. 33–37.—Fig. 33, cross-section through meristem (*m*) of epicotyl. Fig. 34, portion of stem showing differentiation of procambium. Fig. 35, cross-section of stem through region of vascular bundle at time of protoxylem differentiation. Fig. 36, same through vascular bundle. Fig. 37, same through median bundle to leaf.

The relationship between the sieve tubes and companion cells is similar to that in the root (fig. 14).

Summary

1. The root of the soy bean is developed according to JANCZEWSKI'S "fourth angiospermous type," and at maturity is a tetrarch radial protostele which, as COMPTON (**2**) and DE CANDOLLE (**1**) point out, is common to many Leguminosae and especially to the Phaseoleae of which the soy bean is a member. A conical mass of parenchymatous cells comprises the root cap. The epidermis consists of a single layer of cells from which root hairs arise. The cortex is composed of several layers of parenchymatous cells and the endodermis and pericycle are each a single layer. Secondary roots arise from the pericycle. The anatomy of the secondary root is similar to that of the primary root except that usually only one large metaxylem vessel is matured in the center of the root instead of several. The protoxylem of the primary root consists of one to three spiral-reticulate vessels. The metaxylem matures in two stages and differentiates as pitted vessels and parenchyma. Part of the primary phloem is crushed at the initiation of cambial activity while the remainder differentiates as fibers and groups of sieve tubes and companion cells interspersed with parenchyma. A sieve tube may be the same length as the companion cell or may equal two companion cells in length.

2. Root-stem transition in the Phaseoleae is either low or intermediate, being low in the soy bean. As they pass upward the four exarch radial vascular groups of the root bifurcate and reorient as eight endarch collateral bundles.

3. The double traces of the cotyledons pass downward and converge against two vascular groups of the root in the same plane.

4. The cotyledons are fleshy photosynthetic leaves which drop off after maturation of the first pair of opposite leaves.

5. The first leaves are simple and opposite with a pair of stipules at the base of the petiole. The trace to each leaf is composed of three bundles which are differentiated downward against the vascular system of the root at the level of transition.

6. The later leaves are alternate and compound and their traces, consisting of three bundles, pass downward through two or more internodes before anastomosing with the lateral traces of other leaves.

7. The stem consists of an endarch collateral dictyostele, peri-

cycle, cortex, and epidermis. The primary vascular skeleton is a dictyostele consisting of the common bundles of the leaf traces. Usually all the vessel segments have the annular and spiral thickening of protoxylem but sometimes a few metaxylem vessels with pitted walls can be observed. A large part of the primary xylem remains parenchymatous and later matures as connective tissue. Primary phloem consists of sieve tubes and companion cells separated by parenchyma. They have the same histology as in the root. The pericycle immediately external to the vascular bundles differentiates as fibers but in the region of the medullary rays remains parenchymatous.

The writer takes pleasure in expressing his indebtedness to Dr. H. E. HAYWARD, who has given valuable suggestions and criticisms during the progress of this work.

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STEM STRUCTURE OF GRASSES ON THE JORNADA EXPERIMENTAL RANGE

R. H. CANFIELD

(WITH EIGHT FIGURES)

Introduction

The orthodox description of the grass family contains the phrase "stems usually hollow," which has been generally accepted as meaning that all mature grasses other than the genera of the Andropogoneae and Nazieae have hollow stems. Early in 1927, when certain grasses not members of either of these tribes were examined, they were found to have solid stems. Continued investigation showed that a very high percentage of the mature grasses native to the semi-arid Jornada plain have solid internodes.

BEWS (1) states that the stems of grasses are usually hollow, but that a number of "types" have solid stems, including the Andropogoneae and many of the Paniceae. VAVILOV (6) also refers to a solid stemmed variety of *Agropyrum cristatum* Beauv., which he reports as being widely distributed over European and Asiatic Russia.

JORNADA EXPERIMENTAL RANGE.—This area is a branch of the Southwestern Forest and Range Experiment Station, and includes approximately 300 square miles of semi-arid lands located near Las Cruces, New Mexico, some 50 miles north of the Mexican border. The region is typical of the south-central New Mexico grasslands. The greater part of it is a flat to slightly rolling mesa with elevations ranging from 4100 to 4700 feet. In addition, there are about 73 square miles of mountainous country which include the foothills and western slopes of the San Andres Mountains. Elevations vary in this area from 4700 in the foothills to 8000 feet on the higher peaks.

CLIMATE.—Climatically the Jornada region is one of the most arid in the southwest. The average annual rainfall for the 17-year period covered by the Jornada Experimental Range precipitation records is 8.99 inches, with a maximum of 17.73 inches and a minimum of 3.54 inches. Annual rainfall for seven of the seventeen

years was above the average for the period, and for ten of the years was below the average.

Usually the first effective summer rains fall late in June or early in July. A general beginning of plant growth takes place after this rainfall and continues through a season of about 100 days. This period, which includes July, August, and September, is commonly referred to as the growing season. According to the Jornada records the average precipitation for the period of growth is 4.64 inches. Of the seventeen years included, six were above the average and eleven were below it. During this period of record the maximum summer seasonal was 8.53 inches and the minimum was 2.34 inches.

The average date of the last spring frost is April 9, and the average date of the first fall frost is October 26; 200 days is the average frostless period. Temperatures as high as 106° F. and as low as −8° F. sometimes occur. The average wind velocity is 7.1 miles per hour.[1]

Recent measurements made at the Jornada headquarters show that a 0.5-inch daily evaporation loss from a free water surface is common during the summer and early fall months. A combination of hot dry winds and low relative humidity, sometimes as low as 10 per cent, gives a high rate of evaporation and a correspondingly high rate of transpiration in the vegetation.

The most prominent climatic characteristic is of course the ever-recurring periods of insufficient soil moisture. SHANTZ (5) states: "True drought can occur only when the available soil water has been exhausted." It is also accepted as fact that the individual plant represents the sum of all the factors which have affected its growth, of which available water is one. Drought is therefore a specific condition which can be detected in observable effects on the vegetation. The character of the effects from true drought are such as would, if continued for a sufficient time, eliminate the plants concerned from the area under consideration.

The writer (3) has previously offered a definition of drought which is believed to be sufficiently precise for range purposes. Drought was defined as a condition in which the water requirements of the established dominant plants of the highest successional stage exceed

[1] The climatic data are taken from the records of the New Mexico A. & M. College, located 20 miles south of the Jornada Experimental Range.

the available water supply, and which, if continued beyond the life span of these plants and beyond the duration of the viability of their seeds, would result in the extinction of the species. Drought in the Jornada region may be separated into two classes, general and stratified. General drought affects the whole of the soil profile utilized by plants; stratified drought occurs in horizontal layers within the soil profile and affects only the plants which have their roots mainly in the dry layer.

In connection with the climatic conditions, the sand storms which occur with unfailing regularity during the spring and early summer months are of importance. In addition to the damage incident to erosion and deposition of soil, the abrasive action of the sand blast increases the difficulties under which the succulent vegetation makes its struggle for an existence.

VEGETATION.—In spite of the climatic conditions, the Jornada grasslands produce abundant crops of forage. Figures 1 and 2 show typical range conditions in a year of average seasonal rainfall. The native vegetation consists of herbaceous plants and shrubs which are capable of maintaining an existence under conditions of low rainfall, high temperature, and a dry atmosphere. The more common plants take advantage of periods of abundant moisture, either by completing their growth in one usually short, wet period or by alternating rapid growth with dormancy through the succeeding wet and dry periods until maturity is attained.

CANFIELD (4) separates the grasses occurring in this region into four divisions or classes, based on the order of their development. The influence of the stem structure is apparent in these groups.

One class is composed of the annuals which complete growth early in the season, during a period of available moisture when the temperatures are low and the relative humidity is high enough to retard transpiration. The proportions of grasses with solid and with hollow stems are about equal in frequency in this group; nevertheless the solid stemmed species contribute materially to the plant cover while the hollow stemmed species occur infrequently.

A second group, composed chiefly of the perennial *Aristida* and *Hilaria mutica*, produce a mature crop of plants at any time during the frostless season when an unbroken period of available moisture

of sufficient length occurs. A spring crop of these grasses followed by one or more summer crops is not an uncommon event. The members of this group are solid stemmed without exception.

A third group, represented by *Bouteloua eriopoda* and species of *Sporobolus* and *Muhlenbergia*, occurring on the well drained sandy or

FIGS. 1, 2.—Fig. 1 (above), range in early summer before growing season begins. Fig. 2 (below), range in fall after a good growing season.

gravelly soils, make their growth by stages. These grasses develop rapidly during periods of abundant moisture and high night temperature, although usually they are unable to attain maturity in one unbroken period. In the droughts which usually intervene between the rains, these grasses suspend activity and remain in a dormant condition, bursting into growth when the next soil-saturating rain

comes. An almost immediate response to moisture is an outstanding characteristic of this group. Two members of the third group, *Bouteloua eriopoda* and *Muhlenbergia porteri*, are notable because of their true perennial habit of growth. Stems of these grasses remain green throughout the winter and leaf out again the following year. All species falling into this group are solid stemmed.

A fourth group contains *Koeleria cristata* and other grasses, most of them hollow stemmed, which are nearing the lower limits of their ranges. These species occur only in the more favorable situations, such as depressions, intermittent stream beds, and sheltered spots where moisture is present for longer than the usual periods.

Artificial planting of foreign grasses has resulted in failure. Grasses from distant arid lands which come highly recommended have not survived a single season.

Investigation

FIELD METHODS.—Field investigations on the stem structure of grasses were started in the late summer of 1927, and carried through each succeeding summer and fall up to and including the 1930 seasons. Mature plants of the various species were selected and a well developed culm was taken from the specimen. The culms were cut transversely through at a point about equidistant from two nodes, and an examination made with a hand lens. Should a stem be of very small diameter, or if for any other reason there was doubt as to the determination of the nature of the structure with the hand lens, material was selected in the field and preserved for microscopic study in the laboratory.

Identification of the species was established by making a study of each grass and comparing specimens collected in the field with authentic specimens in the Jornada Experimental Range herbarium. All specimens in this herbarium have been identified by the United States Bureau of Plant Industry.

LABORATORY METHODS.—Material collected in the field for laboratory use was immediately placed in a small individual vial of formalin acetic alcohol. The vial was temporarily labeled and numbered in the field. Later each specimen received a permanent label, placed inside the vial, which was then corked and sealed with paraffin.

Considerable difficulty was encountered in preparing the material for imbedding and sectioning. The high silicon content of the grass stems was a serious obstacle which prevented their successful sectioning. In attempts to remove the silicon, the material was immersed in solutions of hydrofluoric acid of different concentrations for varying periods of time. At last it was found that the use of 100 per cent hydrofluoric acid with an immersion period of one week was about right for the greater part of the material.

Further difficulties developed in sectioning. Good sections could not be secured by the usual alcohol stages of dehydrating and the usual paraffin method of imbedding. When the material was run through the alcohols using a 5 per cent solution as a beginning and advancing by 5 per cent stages to absolute, however, the result was satisfactory. The stems were then imbedded in celloidin.

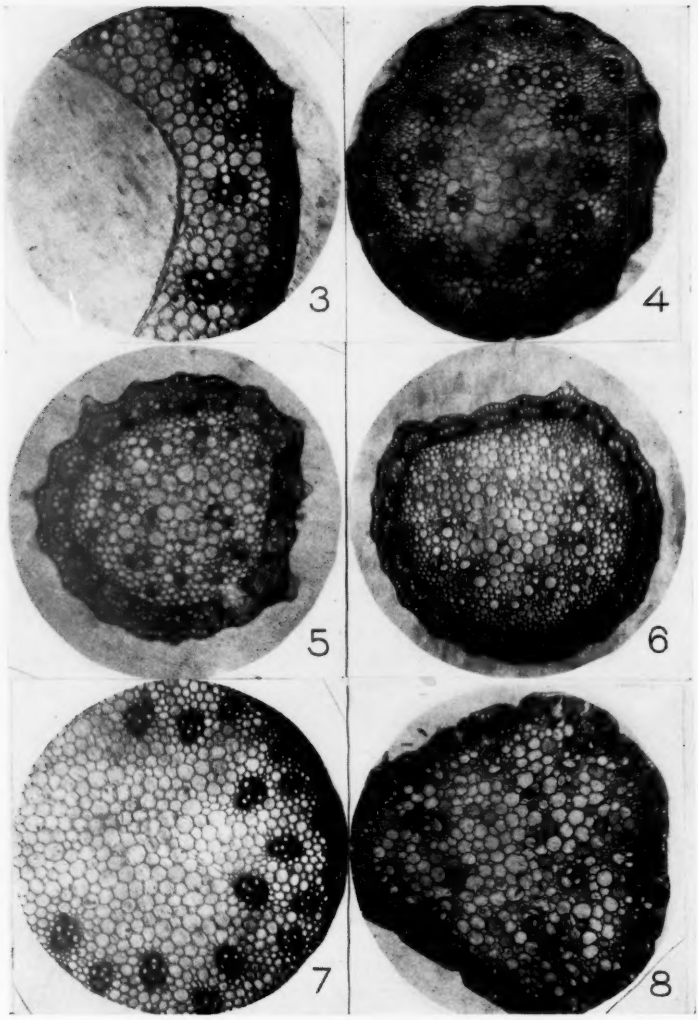
The formula used in making transverse sections of grass stems is as follows:

1. Kill in formalin acetic alcohol solution.
2. Immerse in 100% HF one week.
3. Wash in running water 24 hours.
4. Dehydrate using alcohol stages 5% to 100%, 5% in each step, changing at 4-hour intervals.
5. Imbed in celloidin, 2% to 16%, 12 hours in each solution.
6. Stain sections in iron-alum haematoxylin.
7. Mount in thin balsam.

Figures 3–7 show the stem structure of a few representative specimens of solid and hollow stemmed grasses. The cross-sections were made at a point about equidistant from two nodes and are typical of the species which they represent. In making the slides from which the photomicrographs were taken, it was necessary to cut the sections much thicker than usual to avoid tearing the thin walled cells in the central portion of the stems. These cells had become fragile from exposure to the strong acid used in removing the silicon.

Since it was desired that the photographs show the cell structure in the central parts of the stems, it was necessary in most instances to throw the heavy walled cells of the outer portions of the stems out of focus.

No attempt is made in this paper to enter into a detailed discus-



FIGS. 3–8.—Transverse sections of grasses: Fig. 3, *Leptochloa fascicularis*, hollow stemmed annual grass occurring only in favored locations where moisture is available for longer than the usual periods. Note large central cavity. Fig. 4, *Hilaria mutica*, solid stemmed perennial grass (solid stems would be expected in this genus) occurring only on heavy clay soils and thriving only on areas subjected to periodic summer flooding. Fig. 5, *Scleropogon brevifolius*, perennial grass described by CAMPBELL (2) as a pioneer occurring on denuded clay flats. Fig. 6, *Muhlenbergia porteri*, perennial grass once common but now sparse, probably because stems are perennially green and therefore much sought by grazing animals. Fig. 7, *Sporobolus cryptandrus*, solid stemmed perennial grass occurring on dry sandy ridges and plains. Fig. 8, *Cyperus uniflorus*, a sedge requiring moist or wet soil as its habitat, included here to show similarity in stem structure to grasses of the semi-arid mesas.

sion of the differences in cell structure and cell arrangement which are apparent under the microscope. However, the thin walled cells of the central portions of the solid stemmed grasses and the arrangement of the vascular bundles are interesting items.

In the following list of species, all the grasses known to occur on the Jornada Experimental Range have been classified as to whether the stems are solid or hollow.

SOLID STEMMED ANNUALS (7 SPECIES)

Nazieae, Tribe III
- Nazia aliena (Spreng.) Buckl.
- N. racemosa (L.) Kuntze

Paniceae, Tribe V
- Cenchrus pauciflorus Benth.

Agrostideae, Tribe VIII
- Aristida adscensionis L.

Chlorideae, Tribe X
- Bouteloua aristidoides (H.B.K.) Griseb.
- B. barbata Lag.
- B. parryi (Fourn.) Griff.

HOLLOW STEMMED ANNUALS (8 SPECIES)

Paniceae, Tribe V
- Panicum barbipulvinatum Nash
- P. hallii Vasey
- P. hirticaule Presl.

Chlorideae, Tribe X
- Leptochloa fascicularis A. Gray (fig. 3)

Festuceae, Tribe XI
- Eragrostis pilosa (L.) Beauv.
- Festuca octoflora Walt.
- F. octoflora hirtella Piper
- Munroa squarrosa (Nutt.) Torr.

SOLID STEMMED PERENNIALS (44 SPECIES)

Andropogoneae, Tribe II
- Andropogon saccharoides Swartz

Nazieae, Tribe III
Hilaria mutica (Buckl.) Benth. (fig. 4)
Paniceae, Tribe V
Echinochloa crus-galli (L.) Beauv.
E. crus-galli mitis (Pursh) Peterm.
Panicum obtusum H.B.K.
Paspalum distichum L.
Chaetochloa macrostachya (H.B.K.) Scribn. & Merr.
Agrostideae, Tribe VIII
Aristida divaricata Humb. & Bonpl.
A. glauca (Nees) Walp.
A. havardii Vasey
A. longiseta robusta Merr.
A. pansa Woot. & Standl.
A. purpurea Nutt.
A. schiediana Trin. & Rupr.
Epicampes emersleyi (Vasey) Hitchc.
E. rigens Benth.
Lycurus phleoides H.B.K.
Muhlenbergia arenicola Buckl.
M. monticola Buckl.
M. pauciflora Buckl.
M. porteri Scribn. (fig. 6)
M. repens (Presl.) Hitchc.
Oryzopsis hymenioides (Roem. & Schult.) Ricker
Sporobolus airoides Torr.
S. asperifolius (Nees & Meyen) Thurb.
S. auriculatus. Vasey
S. cryptandrus (Torr.) A. Gray (fig. 7)
S. flexuosus (Thurb.) Rydb.
S. giganteus Nash
S. nealleyi Vasey
Stipa eminens Cav.
Chlorideae, Tribe X
Bouteloua breviseta Vasey
B. curtipendula (Mich.) Torr.
B. eriopoda Torr.

B. gracilis (H.B.K.) Lag.
B. hirsuta Lag.
Chloris virgata Sw.
Leptochloa dubia (H.B.K.) Nees
Festuceae, Tribe XI
Distichlis spicata (L.) Greene
Eragrostis cilianensis (All.) Link
E. erosa Scribn.
Scleropogon brevifolius Phil. (fig. 5)
Triodia mutica (Torr.) Scribn.
T. pilosa (Buckl.) Merr.

HOLLOW STEMMED PERENNIALS (10 SPECIES)

Paniceae, Tribe V
Leptoloma cognatum (Schult.) Chase
Valota saccharata (Buckl.) Chase
Agrostideae, Tribe VIII
Agrostis verticillata Vill.
Muhlenbergia rigida (H.B.K.) Kunth
Stipa columbiana Macoun
S. scribneri Vasey
Aveneae, Tribe IX
Koeleria cristata (L.) Pers.
Chlorideae, Tribe X
Capriola dactylon (L.) Kuntze
Festuceae, Tribe XI
Pappophorum wrightii S. Wats.
Triodia pulchella H.B.K.

Discussion

The terms solid stemmed and hollow stemmed refer to the condition of the grass stems at maturity. Due consideration has been given to the natural condition of the growing stem, and only mature stems have been used in this study. It is a recognized fact that nearly all grass culms have no hollow space in the internodes during the early periods of growth. This condition is especially true of the meristematic regions just above the nodes.

This study of the Jornada grasses includes seven tribes which are represented by 30 genera and 69 species. Of the total number, 51 species (74 per cent) have solid stems while only 18 species (26 per cent) have hollow stems.

ANNUAL GRASSES.—The annual grasses consist of 15 species of which seven are solid stemmed and eight are hollow stemmed. Annual grasses, because of their scattered occurrence and their short stature, constitute only a small fraction of the Jornada forage crop.

The solid stemmed annual species make their growth at any time during the growing season when the top layer of soil furnishes moisture for a period of sufficient length for them to complete development. These grasses belong in the class which completes growth in one unbroken interval. Although the rate of development may be perceptibly slowed down at times, either by a decrease in the depth of the moist top layer of soil or by an increase in the rate of transpiration to a point which is almost equal to the rate of absorption, in no observed instance can these grasses be said to have passed through a period of dormancy and to have resumed their growth with the return of favorable conditions.

The hollow stemmed annual grasses rely on a rapid development from germination to maturity for the maintenance of the species. Their period of growth generally is restricted to a short rainy interval in the early part of the season, at which time the days are cool and the relative humidity is high. However, an occasional plant may be found later in the season in sheltered spots or in catch basins, where the required climatic conditions are maintained by unusual factors beyond the regular season.

There is overwhelming evidence in practically all the species which have been observed that the hollow stemmed annual grasses of this semi-arid region thrive only under climatic conditions which are somewhat similar to those of the humid regions where the grasses are chiefly hollow stemmed.

PERENNIAL GRASSES.—The perennials are represented by 55 species, of which 45 (82 per cent) are solid stemmed while only 10 species (18 per cent) are hollow stemmed.

Native perennial grasses produce the major part of all the forage

on Southwestern plains. In the Jornada region the species of five genera, *Bouteloua*, *Sporobolus*, *Aristida*, *Hilaria*, and *Scleropogon*, furnish 85 per cent or more of the forage derived from grasses. All the species representing these genera are able to withstand to a marked degree protracted drought with grazing. They have the ability to exist in the less favorable situations, as well as in the more favorable ones. Observed representatives of these genera native to this region are without exception solid stemmed grasses.

The hollow stemmed perennial grasses furnish very little forage. They are sparsely scattered throughout the region, being restricted to the more favorable sites. Even in these more favorable locations, such as the bottoms of intermittent streams or the higher elevations where precipitation is greater, the hollow stemmed species generally exist under the protection of some jutting rock or in the shade of some hardy shrub.

Summary

1. In the species of grasses observed, the types of stem structure in the Jornada Range grasses apparently reflect the moisture requirements and the periods of growth.

2. The solid stem is characteristic of the grasses which are apparently best able to survive under the semi-arid conditions of the Jornada region.

3. Almost three-fourths (74 per cent) of the grasses collected have solid stems.

4. Tribes commonly reported as having solid stems, Andropogoneae and Nazieae, are represented by one genus each.

5. Solid stemmed perennial grasses produce 85 per cent or more of the forage.

6. Hollow stemmed grasses either grow only in the more favorable locations or escape drought by completing their growth during a few weeks of highly favorable moisture conditions.

7. Hollow stemmed grasses have not the ability to withstand the long dry periods.

8. There are strong indications that the solid stem is an index which may be employed in the selection of grasses for introduction into semi-arid regions.

The writer acknowledges his indebtedness to Mrs. AGNES CHASE, of the Bureau of Plant Industry; to W. A. DAYTON, of the U.S. Forest Service; to Dr. H. H. CASTLE, of Yale University; and to the Southwestern Forest and Range Experiment Station.

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OVULE AND EMBRYO SAC OF PLUMBAGO CAPENSIS

ARTHUR W. HAUPT

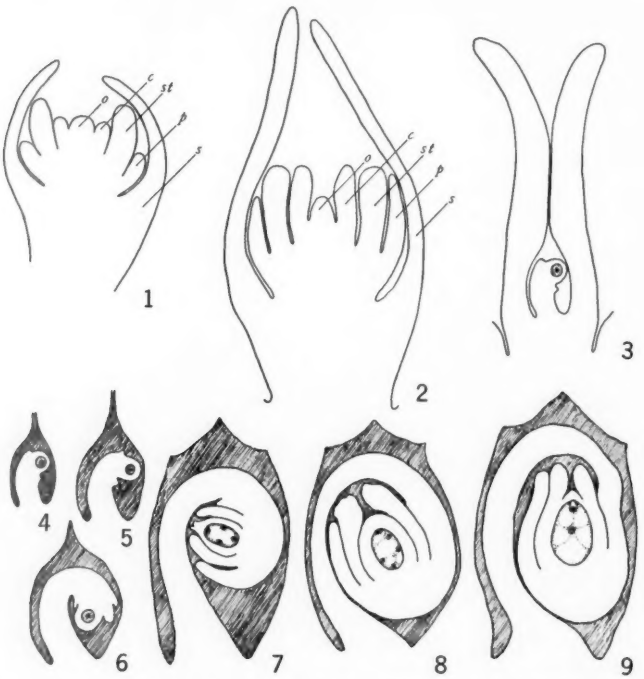
(WITH TWENTY-EIGHT FIGURES)

Introduction

Plumbago capensis is a South African shrub cultivated in greenhouses and extensively grown in the open in southern California. It produces a great profusion of azure blue or white flowers throughout the summer and autumn. Flowers in all stages of development may be collected from the same plant at any time during the blooming period, as new spikes continue to be formed. Although interest in the present investigation was confined to the ovule and embryo sac, it was found unnecessary to remove the ovule from the ovary, or even to remove the ovary from the flower, in order to prepare it for sectioning in paraffin. Whole flower buds, in the case of the smallest ones, were dropped into the fixing fluid, while in handling larger buds and opening flowers, the upper portions of the calyx and corolla were cut away so as to facilitate the entrance of the fixing fluid. The fixing agents giving the best results were formalin-acetic-alcohol and Nawaschin's fluid.

The only previous investigation of importance on the Plumbaginaceae is that of DAHLGREN (1), who in 1915 reported in a preliminary announcement the discovery of a new type of embryo sac in *Plumbagella*. This was followed the next year (2) by a full account of studies made on sixteen species of Plumbaginaceae, representing the following genera: *Acantholimon*, *Armeria*, *Goniolimon*, *Limoniastrum*, *Statice*, *Ceratostigma*, *Plumbago*, and *Plumbagella*. Of these, the first five genera, belonging to the Staticeae, were found by DAHLGREN to exhibit a normal 8-nucleate embryo sac of the *Lilium* type, while the last three genera, belonging to the Plumbagineae, were all found to possess a 4-nucleate sac of a unique and hitherto undescribed type. He investigated *Ceratostigma plumbaginoides*, *Plumbagella micrantha*, and three species of *Plumbago*, *P. capensis*, *P. pulchella*, and *P. zeylanica*. *Plumbagella* was studied in greatest detail.

I have examined only *Plumbago capensis*, but have made a thorough study and can state positively that this species does not exhibit the kind of embryo sac said by DAHLGREN to be characteristic of the three genera of Plumbagineae upon which he worked, but possesses a remarkable type which has not been described in any other angiosperm.



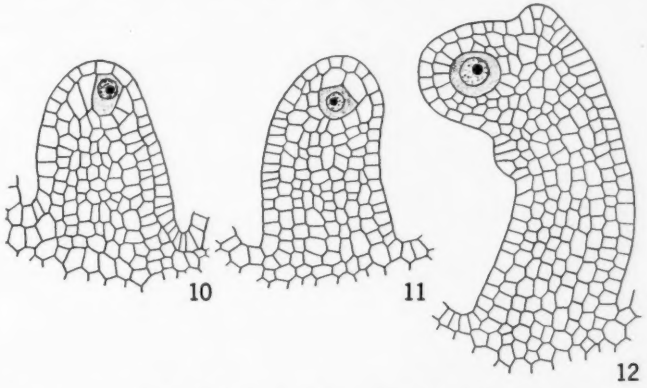
FIGS. 1–9.—Stages in development of ovule: *o*, ovule; *c*, carpel; *st*, stamen; *p*, petal; *s*, sepal. Figs. 1 and 2 are median longitudinal sections of young flower buds. ×80.

Ovule

In general, the present study of the structure and development of the ovule has confirmed the previous work of DAHLGREN (**2**). Judging from such stages as are shown by figures 1 and 2, the floral members seem to arise in acropetal succession. Earlier stages were not available. Following the appearance of the carpels, the apical

meristem of the floral axis becomes transformed directly into the single ovule which is characteristic of the Plumbaginaceae. Thus the ovule is strictly cauline in origin. The carpels develop around the ovule as a zone of tissue with five free tips. At first the style is hollow but later it becomes solid.

The ovule primordium has the form of a conical elevation consisting of uniform undifferentiated cells (fig. 2). At first it grows slowly and evenly, but soon a more rapid growth on one side causes the



FIGS. 10–12.—Longitudinal sections of young ovules showing formation of megaspore mother cell: fig. 10, archesporial cell (shaded); fig. 11, archesporial cell given rise to megaspore mother cell (shaded) and two parietal cells; fig. 12, megaspore mother cell (shaded) overlain by layer of parietal cells. ×400.

young ovule to bend in the opposite direction. The inner integument now arises as an annular outgrowth at the base of the nucellus; and as the curvature of the ovule becomes more pronounced through the elongation of the funiculus, the outer integument appears (figs. 3–6). The ovule is now inverted, the nucellus being directed downward. As development proceeds, however, the curvature of the ovule continues until it has turned completely over, the micropyle pointing upward (figs. 7–9).

The formation of the megaspore mother cell is similar to that described by DAHLGREN (2) for *Plumbago zeylanica*. The archesporium, which is hypodermal in origin, appears while the ovule is still very young, always before the integuments arise (fig. 10). Frequent-

ly the archesporium involves two cells (fig. 27) but as a rule only one is present. Since an archesporium consisting of more than one cell is confined chiefly to certain primitive families of Archichlamydeae and to other isolated cases, its occasional occurrence in *Plumbago* represents a primitive character.

The archesporial cell divides by a periclinal wall to form a primary parietal cell and the megaspore mother cell, an anticlinal division soon taking place in the former (fig. 11). By the formation of additional anticlinal walls, a layer of parietal cells arises beneath the epidermis of the nucellus (fig. 12), and by further periclinal divisions this is soon increased to two layers. In view of the fact that the suppression of parietal tissue in the ovule is nearly universal among the Sympetalae, its occurrence in *Plumbago* is especially noteworthy.

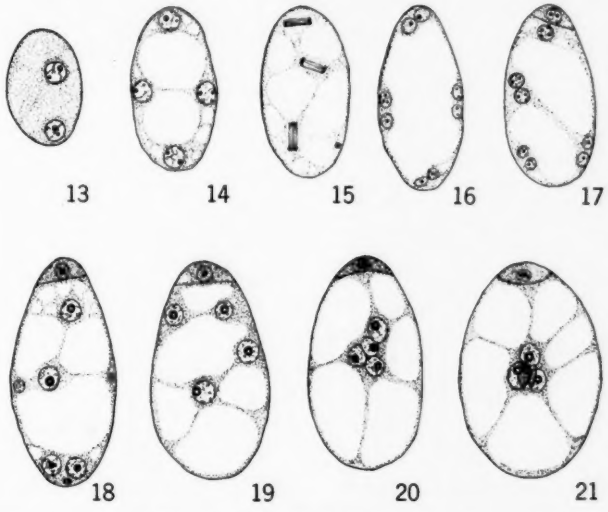
Embryo sac

As DAHLGREN (**2**) found in all of the species of Plumbaginaceae which he investigated, the megaspore mother cell, like that of *Lilium*, develops directly into the embryo sac, no linear tetrad of megaspores being formed. After the mother cell enlarges considerably, its nucleus undergoes the heterotypic mitosis (fig. 13). The homoeotypic mitosis, immediately following, results in the formation of four free nuclei arranged so that one is at each end of the embryo sac and one is at each side (fig. 14). This crosslike arrangement of the megaspore nuclei is very characteristic. By this time the embryo sac has become conspicuously vacuolate. As DAHLGREN found in the three species of *Plumbago* which he studied, no vacuole is formed in the binucleate stage.

Simultaneously the four megaspore nuclei now undergo a division, resulting in the formation of an 8-nucleate embryo sac (fig. 15). The daughter nuclei remain together in pairs, so that their orientation is the same as that shown by the four megaspore nuclei of the preceding stage (fig. 16). Instances of embryo sacs with eight nuclei were not so frequently observed as those with four nuclei, but the finding of several cases like the one shown in figure 16, and of one case where four mitotic figures were present (fig. 15), definitely establishes the fact that an 8-nucleate stage is reached.

The egg of *Plumbago capensis* is organized from one member of the

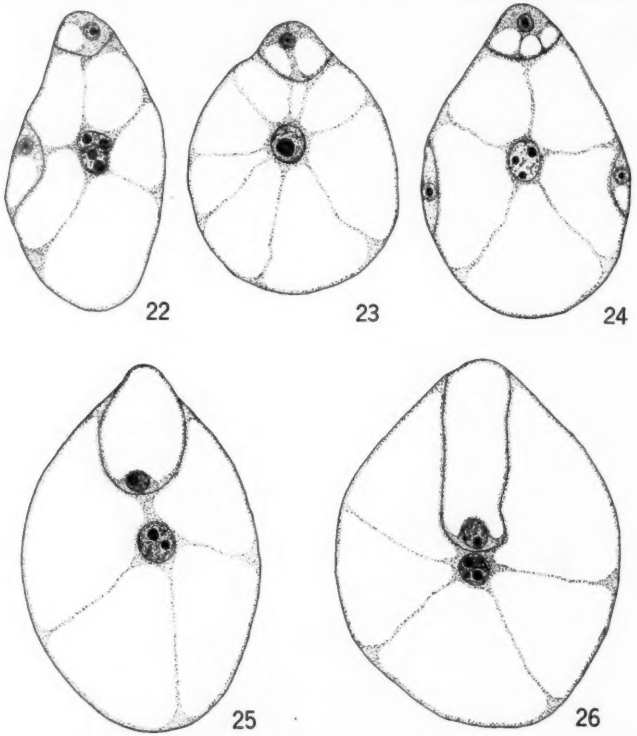
micropylar pair of nuclei present in the 8-nucleate stage, and is thus removed from the megaspore nucleus by only one division. The egg nucleus, together with a small amount of cytoplasm, is cut off from the rest of the embryo sac by a thin membrane (fig. 17). It remains as a small lenticular cell at the micropylar end of the sac until changes have taken place elsewhere. No synergids are differentiated. After the egg is organized, the other seven nuclei are left free in the



FIGS. 13–21.—Early stages in development of embryo sac: fig. 13, 2-nucleate stage; fig. 14, 4-nucleate stage; fig. 15, 4 nuclei dividing (one of the mitotic figures is oriented at right angles to plane in which section was cut); fig. 16, 8-nucleate stage; fig. 17, cutting off of egg; fig. 18, differentiation of the four polar nuclei; figs. 19, 20, fusion of polar nuclei. ×400.

general cytoplasm of the embryo sac. Four of these free nuclei increase slightly in size and gradually approach one another, functioning as polar nuclei, while as a rule the three remaining nuclei degenerate (fig. 18). Embryo sacs containing four large free nuclei and a small deeply staining cell at the micropylar end were very commonly encountered in the preparations (fig. 19), but their interpretation was not possible until stages intermediate between this one and the equally common 4-nucleate stage were found.

The four polar nuclei eventually come in contact near the center of the embryo sac and soon fuse (figs. 20, 21). Many of the preparations showed four nuclei about to unite, and where fewer were seen in close contact it was apparent that some had already fused (fig. 22).



FIGS. 22–26.—Later stages in development of embryo sac, with egg at micropylar end and fusion nucleus in center. In figs. 22 and 24 persistent lateral cells are formed from nuclei which ordinarily disappear in an earlier stage. Fig. 26 shows a mature embryo sac ready for fertilization. ×400.

A single large primary endosperm nucleus in an embryo sac containing an egg still immature is a stage which was frequently encountered (figs. 23, 24). Of the four fusing nuclei, one is the second member of the pair originally present at the micropylar end of the embryo sac,

but it could not be determined with certainty that each of the other three polar nuclei represents a single member of each of the other three pairs present in the 8-nucleate stage. Presumably, however, this is the case.

As previously stated, three of the eight nuclei formed in the embryo sac usually degenerate. Except for their position, they might be said to correspond to the antipodal nuclei of a normal 8-nucleate sac. While the degeneration of these nuclei is the rule, occasionally one, two, or even all three of them may persist and become organized as small naked cells. In figure 22 one such cell is present, while in figure 24 there are two. These persistent nuclei, which always seem to occupy a parietal position, are cut off by a membrane with a small amount of cytoplasm, as occurs when the egg is formed. In fact, often one or more of these cells may later enlarge and become egglike in appearance, suggesting that the embryo sac of *Plumbago* may have been derived phylogenetically from one in which each of the four megaspore nuclei gave rise to one egg and one polar nucleus. This interpretation is suggested by the situation in the Penaeaceae (**6**) and in *Euphorbia procera* and *E. palustris* (**3**, **4**, **5**), where by an additional division four peripheral groups of four nuclei each are formed, each group giving rise to a polar nucleus and to three cells resembling an egg apparatus. As in *Plumbago*, the primary endosperm nucleus results from the fusion of the four polar nuclei.

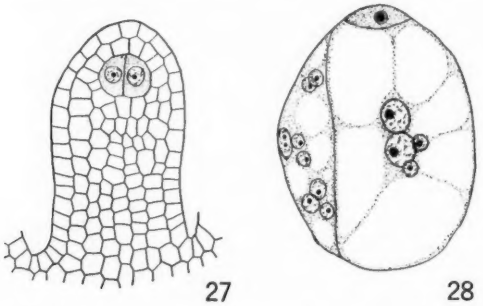
With the fusion of the four polar nuclei consummated, the egg undergoes a marked enlargement. Up to this point the nucleus lies at the upper end of the egg, the cytoplasm being vacuolate below; but with the enlargement of the egg the nucleus moves to its lower end and a large central vacuole fills its greater portion (figs. 25, 26). The fusion nucleus is situated immediately below the egg and rather near the center of the embryo sac. Except in rare cases, the mature embryo sac of *Plumbago capensis* contains only the egg and the fusion nucleus.

Fertilization was not studied, since in none of the flowers sectioned had pollination taken place. This was really an advantage, since it eliminated the possibility of any of the nuclei found in the embryo sac being male. Pollination is an infrequent occurrence, and as a result seeds are seldom formed. The flowers are sometimes visited by hawk

moths, which effect pollination. This was demonstrated by observing that flowers so visited later produced seed while other flowers did not. Artificial pollination is always successful. A number of seedlings were raised from seeds produced in this way. It was thought not necessary to artificially pollinate a number of flowers so that the details of fertilization could be studied, since DAHLGREN (2) has already given a full account of double fertilization, embryogeny, and endosperm formation.

Abnormalities

The presence of two megaspore mother cells is of such frequent occurrence that it can hardly be called an abnormality (fig. 27).



FIGS. 27, 28.—Unusual stages: fig. 27, two megaspore mother cells; fig. 28, two embryo sacs in same ovule. ×400.

Ordinarily only one gives rise to an embryo sac, but rarely both mother cells may function (fig. 28). In such cases one of the embryo sacs is much smaller than the other and somewhat tardy in its development. Several instances were seen of two embryo sacs in the same ovule, but separated from each other by several layers of nucellar tissue; and in one or two cases two ovules, partially grown together, were found in the same ovary. DAHLGREN (2) found a single case of two ovules in the same ovary in all three species of *Plumbago* which he investigated.

Discussion

DAHLGREN'S work was done chiefly on *Plumbagella*, but he found a similar type of embryo sac development in *Plumbago* and in *Cerato-*

stigma. His series of stages up to the 4-nucleate stage is in close agreement with mine, except that vacuolization in *Plumbagella* begins at the binucleate stage. Similarly DAHLGREN found in all three genera that the mature embryo sac possesses only an egg and a fusion nucleus. It is only in the development of the mature sac from the 4-nucleate stage that his account and mine differ.

In all three of the genera of Plumbagineae investigated, DAHLGREN (**2**) found that normally no further nuclear divisions occur beyond the 4-nucleate stage. The nucleus lying nearest the micropyle directly becomes the egg, the one next to the chalaza forms a single antipodal cell which soon degenerates, while the other two, representing the polar nuclei, later fuse to form the primary endosperm nucleus. Synergids are entirely absent. DAHLGREN'S figures 52–58, illustrating this development in *Plumbagella*, are much more convincing than his figures 61–72 of *Plumbago*.

Moreover DAHLGREN found certain "aberrant" cases, in both *Plumbago* and *Ceratostigma*, which he interprets on the basis of the development described for *Plumbagella*, but which would seem to be merely stages in such a developmental series as presented here for *Plumbago capensis*. Thus his figures 68 and 71 show embryo sacs of *P. zeylanica* with an egg and three fusing nuclei. In figure 69 a chalazal megaspore nucleus has divided to form two small nuclei, a large fusion nucleus being present near the egg. In figure 72 the fusion of a small nucleus (which DAHLGREN calls the "antipodal nucleus") with the primary endosperm nucleus is shown. In no case, however, did DAHLGREN find four nuclei fusing, but twice in *Ceratostigma* and once in *Plumbago zeylanica* young embryo sacs were encountered which contained, besides the newly formed egg, four free nuclei. In fact, a single 8-nucleate embryo sac (represented by figure 81) was seen in *Ceratostigma*, the occurrence of which DAHLGREN regards as atavistic. The cases just mentioned, considered by DAHLGREN to be anomalous, seem to indicate on the contrary that the embryo sac of *Plumbago* and *Ceratostigma* contain more than four nuclei and that more than two nuclei enter into the formation of the fusion nucleus.

Especially in *Ceratostigma*, but also in *Plumbago*, DAHLGREN (**2**) found anomalous embryo sacs with egglike cells present in addition to the true egg and the fusion nucleus. In *Plumbagella* such cases

were rare. An egglike cell at the chalazal end of the sac of *Plumbagella* is shown by figure 59, and in *Plumbago zeylanica* by figure 73. Figures 78 and 79 represent embryo sacs of *Ceratostigma*, each with a laterally situated egglike cell which DAHLGREN interprets as an antipodal which has assumed an unusual position and changed appearance. His figure 82 shows a similar case in *P. capensis*, while in figure 75 an egglike cell lies next to the egg as if it were a synergid. All of these cases may be explained as cell formation from persistent nuclei which normally degenerate; they are similar to those represented by figures 22 and 24 of the present paper.

DAHLGREN considers that in all probability the *Plumbagella* embryo sac has been derived by reduction from the *Lilium* type. He found the latter present in all of the genera of Staticeae which he examined. The embryo sac of *Plumbago*, having eight nuclei produced by a megaspore mother cell nucleus, may then represent an intermediate stage in a reduction series leading to *Plumbagella*. The fusion of four polar nuclei in *Plumbago*, while having no counterpart in the *Lilium* type of embryo sac, may have arisen incidental to the failure of the synergids and antipodals to be organized. On the other hand, if the embryo sac of *Plumbago* be regarded as having been derived from a 16-nucleate sac of the kind found in the Penaeaceae (**6**), a possibility which I have already suggested, its relation to the type characteristic of the Staticeae would be rather remote.

Summary

1. The single ovule of *Plumbago capensis* is derived directly from the apical meristem of the floral axis, and thus is strictly cauline in origin.

2. The curvature of the ovule embraces a complete circle, so that the micropyle finally is directed upward.

3. Two integuments are present, but the outer one is not developed on the side of the ovule next to the funiculus.

4. Frequently the archesporium involves two cells, but as a rule only one is present.

5. The archesporial cell gives rise to a primary parietal cell and the megaspore mother cell. The former finally produces two layers of parietal tissue.

6. The megaspore mother cell develops directly into the embryo sac, no linear tetrad of megaspores being formed.

7. By the division of each of the four megaspore nuclei, which exhibit a crosslike arrangement, an 8-nucleate embryo sac is formed, the daughter nuclei remaining together in pairs.

8. From one of the micropylar pair of nuclei the egg is organized. No synergids are differentiated. Of the remaining seven nuclei, three usually degenerate, while four approach one another and soon fuse.

9. Occasionally one or more of the three nuclei which usually degenerate persist and form small naked cells which may later enlarge and become egglike.

10. The mature embryo sac consists normally only of a large egg, vacuolate above, and a primary endosperm nucleus formed by the fusion of the four polar nuclei.

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CHROMOSOME BEHAVIOR IN PINUS BANKSIANA FOLLOWING FERTILIZATION[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 453

J. M. BEAL

(WITH PLATES XIII, XIV)

Introduction

Although considerable work has been done on the development of the gametophytes, the process of gametic union, and the development of the embryo in gymnosperms, relatively little detailed attention has been given to the behavior of the maternal and paternal chromosomes during the divisions immediately following fertilization. While a number of investigators have described certain stages in the early embryonal divisions, these reports are in the main fragmentary and incidental to a more general study of other features. My observations are in such close agreement with those of certain investigators and so contrary to those of certain others, that it will not be amiss to review briefly a few of the reports.

In *Sequoia sempervirens* LAWSON (7) reported a longitudinal splitting of the long V-shaped chromosomes during the first embryonal division, and stated that the daughter halves as they pass to the poles are about half the size of the mother chromosomes from which they are derived. He observed about 32 chromosomes in the young sporophyte as compared with about 16 in the gametophyte. In *Bowenia* he (8) reported that in fertilization there is not a complete union of the gametic nuclei preceding the first embryonal division, but that each parental nucleus organizes a separate group of eight chromosomes. A spindle is developed for each group, and these remain separate not only in this first division, but during the four or five subsequent mitoses. This reported behavior appears to be unique in the plant kingdom.

BLACKMAN (1) found in *Pinus sylvestris* that the chromosomes in

[1] This investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

the first division of the zygote fall roughly into two groups, each chromosome of which splits longitudinally during the first embryonal division.

In what is doubtless the most critical and thorough work yet done in the gymnosperms, FERGUSON (**4, 5**) described gametic union and early embryonal divisions in *Pinus strobus*. She observed the condensation of separate groups of chromosomes from the chromatin of each gametic nucleus following fertilization. These two groups of chromosomes remain separate and distinct while the multipolar polyarch spindles are being formed. The spindles then merge into a multipolar diarch spindle and the chromosomes become so arranged in the equatorial region of the spindle that the maternal and paternal elements can no longer be distinguished. She obtained one preparation in which a polar view of an equatorial plate stage showed 24 chromosomes (**5,** fig. 235). Twelve chromosomes were observed in the egg nucleus and it was estimated that the sperm brought in the same number. Her figures 236–238 all show clear indications of a longitudinal splitting of the chromosomes during the first embryonal division. In connection with figure 237 she states, "While this opening [the separation of the daughter chromosomes at the points of the spindle fiber attachments] is still inconspicuous, the two halves of a given chromosome become distinct throughout the entire length of the segment."

All the reports just cited agree that the maternal and paternal chromosomes split longitudinally during the first embryonal division, and that the diploid number is present as the result of gametic union of two haploid gametes.

In *Abies balsamea* HUTCHINSON (**6**) reported that following the fusion of the gametic nuclei two groups of chromosomes are formed separately in the prophases of the first division of the zygote, each group containing the haploid number of chromosomes. "As the two spindles unite the chromosomes become paired, at first approximating side by side, but soon twisting about one another and jointly looping into the form of a C." He states that this is a pairing and not a longitudinal splitting of the chromatin elements, and that the number of pairs is haploid. "Also, the twisting of the chromosomes about one another is identical with their behavior in what is generally re-

garded as a pairing during the prophase of the first reduction division." The bending into C- or V-shaped forms is followed by a transverse segmentation of each component of a pair at the angle of the bent chromosomes. These resulting segments, 4n in number, separate during the metaphases so that each daughter nucleus receives one-half of each of the paired elements. In the second division each of the half chromosomes splits longitudinally and separates as in a regular mitosis.

In *Stangeria paradoxa* CHAMBERLAIN (3) reported "the number of chromosomes, as counted at the equatorial plate stage of the first division of the fertilized egg, is 12; but later free nuclear divisions and also mitoses in root tips show that 24 is the diploid number; further, the mitosis at the formation of the ventral canal nucleus and egg shows 12 chromosomes, proving that this is the haploid number." He found four zygotes undergoing the first mitosis, all in the equatorial plate stage. "In all of these the number of chromosomes is 12, but the double character of the chromosomes is evident; and since 12 and 24 are the haploid and diploid numbers, I believe that there is a pairing of chromosomes at fertilization, as described by HUTCHINSON for *Abies*." No figures are given.

Among the angiosperms a single report of "pairing" between maternal and paternal chromosomes, followed by a transverse break during the first embryonal division, has been reported by WENIGER (9) for two species of *Lilium*. She gives three figures and one diagram, but her report is in disagreement with the findings of all other investigators for the angiosperms.

According to the chromosome theory of heredity, each chromosome carries a series of genes arranged in linear order. In fertilization two sets of chromosomes and hence two lots of genes are brought into association. Except for occasional and apparently rather rare upsets, the chromosomes contributed by the two gametic nuclei are maintained unchanged through equational division in the first embryonal as well as in all subsequent mitoses, the daughter chromosomes being distributed in equal numbers to the resulting daughter nuclei.

Because the type of division described in these three reports is so fundamentally at variance with the generally accepted ideas of

chromosome behavior and genetic theory, a reinvestigation of *Pinus* has been undertaken with the hope of clearing up some of the points at issue.

Material and methods

This report is based on a study of material of *Pinus banksiana* Lamb. The first collections were made in the spring of 1932 near Miller, Indiana, and fertilization was found to occur early in June. During 1933 additional fixations were made three times daily, from June 6 to 16 inclusive. Material was fixed at the time of collection, which was between 9:30 and 10:30 A.M. The carpellate scale was carefully pulled from the strobilus and the adaxial face of each ovule was sliced off so as to expose the megagametophyte directly to the fixing solution. The ovule was then cut or pulled from the scale and dropped quickly into the solution. Short pieces of branches bearing carpellate cones were taken from the trees, placed in a vasculum containing moistened paper towels, and brought into the laboratory, where fixations were made between 2:30 and 3:30 P.M. and again at 8:30 to 10:00 P.M. In every case the collections were made from more than one tree.

Several fixing solutions were employed, but the best results were obtained through the use of a modified Navashin's solution consisting of solution A (1 gm. chromic acid crystals, 7 cc. glacial acetic acid, and 92 cc. distilled water) and solution B (30 cc. formalin and 70 cc. distilled water). Equal volumes of the solutions were mixed just before using. Sections were cut at thicknesses ranging from 10 to 15 μ and stained in iron-alum haematoxylin.

Observations

First embryonal division

In agreement with Blackman (1), Ferguson (4, 5), Chamberlain (2), and other investigators it has been observed that the gametic nuclei of *Pinus banksiana* unite while they are both in the reticulate condition. Shortly afterward the reticula condense to form two groups of chromosomes which can be distinguished as having originated from the respective parental nuclei. A multipolar spindle then develops for each group, apparently independently at first, but soon the two spindles merge into a single multipolar diarch spindle

at the equator of which the chromosomes come to lie. The maternal and paternal chromosomes can now no longer be distinguished from one another (figs. 1 *a-d*). These four figures were taken from adjacent sections of a single nucleus which had been cut at a thickness of 10 μ and represent the equatorial plate stage during the first embryonal division. Several of the chromosomes have been cut in sectioning, but there are sufficient numbers of uncut ones to show the forms and structures characteristic of this stage. It is possible to count more than 20 chromosomes, and in all probability the number is 24, corresponding to the somatic or diploid number. None of the chromosomes gives indications of paired and twisted maternal and paternal elements. Further, the number of "fiber-attachment points" as shown by the drawings is approximately 24 instead of 12, thus confirming the presence of the diploid number of chromosomes at this stage. Some of the chromosomes have evidences of a longitudinal split.

Figures 2 and 3 are taken from adjacent sections of the same nucleus during a metaphase stage. They show the characteristic appearance of the chromosomes as observed in several nuclei during these stages. In agreement with FERGUSON'S (**5**) observations, the chromosomes at this stage show a separation of the daughter halves, not only at the fiber attachment points, but the two halves of a given longitudinally split chromosome are distinct throughout the entire length of each element. Although these two figures do not include the entire complement of chromosomes, more than 12 are present.

During early anaphase (fig. 4) the chromosomes are in the form of U's, narrow U's, and V's. An occasional one has one arm slightly shorter than the other, due to the fiber attachment point being submedian. No case of terminal or subterminal fiber attachment has been seen. More than 12 chromosomes are present in each group, counting the entire U or V as a single chromosome.

In a late anaphase stage (fig. 5) the form and appearance of individual chromosomes is essentially the same as in figure 4. In none of this material is there any evidence of a "pairing" of parental chromosomes nor is there any indication of a transverse segmentation at any of the stages in this division. The length of the chromosomes remains approximately constant throughout the process of

division, while the volume of the daughter halves as they pass to the poles appears to be about half that of the mother chromosome before it has split and separated in the metaphases.

Second embryonal division

In the second embryonal division the two daughter nuclei pass through essentially the same stages as did the nucleus of the zygote during the first embryonal division.

In the equatorial plate stage (fig. 6) the chromosomes have approximately the same general forms as in the corresponding stage in the first division, and they are of similar size in thickness and length. Only a portion of the whole complement is shown here, but counts made on this and adjacent sections indicate 24 chromosomes to be present, thus agreeing with the count determined for the preceding division.

During the metaphases (fig. 7) the chromosomes again have the same form and appearance as in similar stages of the first division. The manner of separation at the fiber attachment points and the indications of longitudinal splitting are practically identical in the two divisions.

Observations of the next two divisions have been made, but since they are so similar to corresponding stages in the preceding divisions it is unnecessary to figure or describe them.

Summary

1. During the first embryonal division in *Pinus banksiana* two groups of chromosomes and two spindles are developed. The spindles quickly merge into one multipolar diarch spindle, upon the equator of which the chromosomes come to lie in one group in which the maternal and paternal elements are no longer distinguishable from one another. Each chromosome splits longitudinally and the daughter halves pass to the poles, 24 chromosomes going to each pole. The second and subsequent embryonal divisions are similar to the first. There is no "pairing" between maternal and paternal chromosomes, nor any transverse segmentation during any of the divisions observed.

2. The only place where such interpretation seems possible is in the anaphases when the daughter chromosomes show the form of

U's and V's, so narrow or pointed at the place of attachment of the spindle fibers that the chromosomes may appear to have undergone or be about to undergo transverse segmentation. When these forms, however, are correlated with the entire series of observable stages which the chromosomes undergo during the first and subsequent embryonal divisions, this interpretation seems scarcely possible.

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EXPLANATION OF PLATES XIII, XIV

All the figures were drawn at table level with the aid of an Abbé camera lucida under a Zeiss apochromatic objective, 2 mm. N.A. 1.40, with compensating ocular 10×. Magnification is approximately 1600×.

PLATE XIII

FIG. 1 *a–d*.—Entire chromosome complement of an equatorial plate stage of first embryonal division. Fiber attachment points indicate the number of individual chromosomes present.

FIGS. 2, 3.—Metaphases from adjacent sections of the first embryonal division showing fiber attachment points and longitudinally split chromosomes.

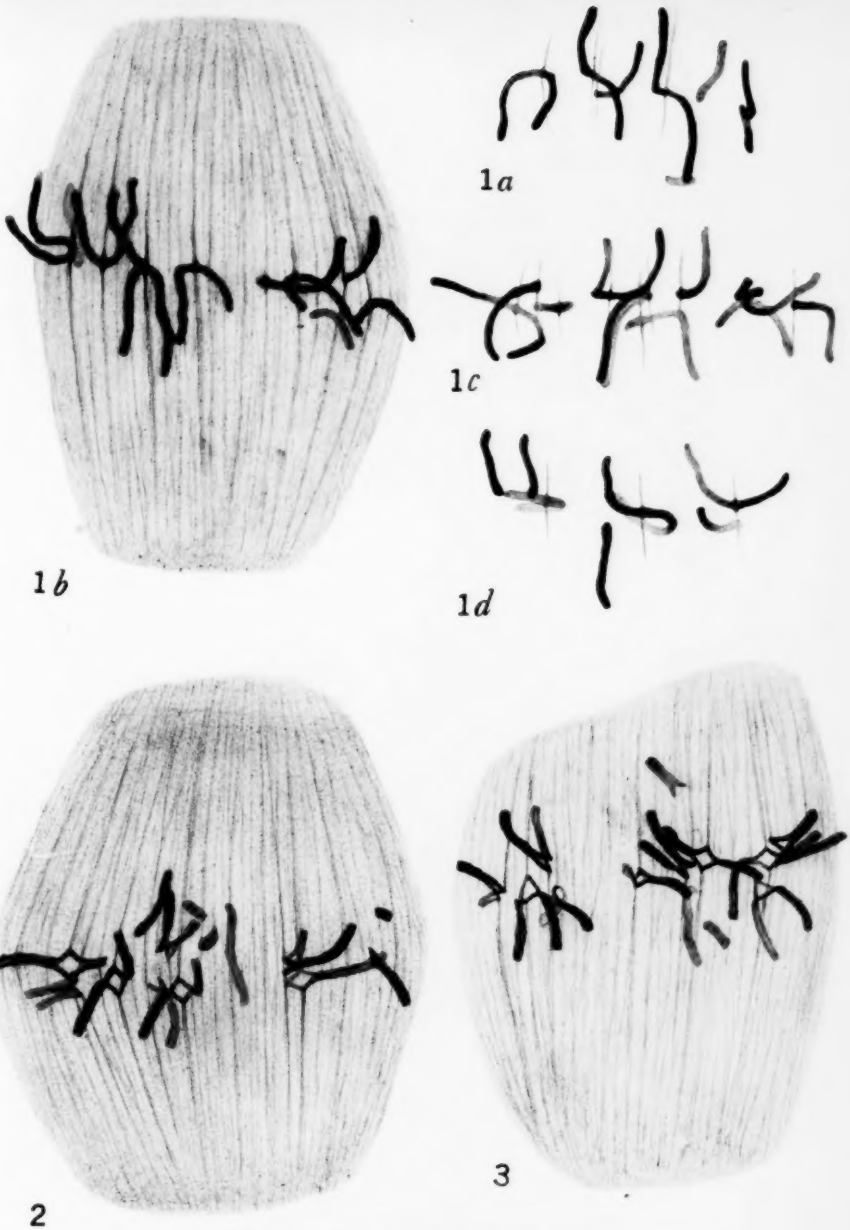
PLATE XIV

FIG. 4.—Somewhat early anaphase of first embryonal division showing typical forms of daughter chromosomes.

FIG. 5.—Late anaphase of first embryonal division.

FIG. 6.—Equatorial plate stage of second embryonal division.

FIG. 7.—Metaphase of second embryonal division.



BEAL on CHROMOSOME BEHAVIOR

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BEAL on CHROMOSOME BEHAVIOR

ANATOMY OF THE XYLEM OF PSEUDOLARIX

ALAN S. PEIRCE

(WITH SIXTEEN FIGURES)

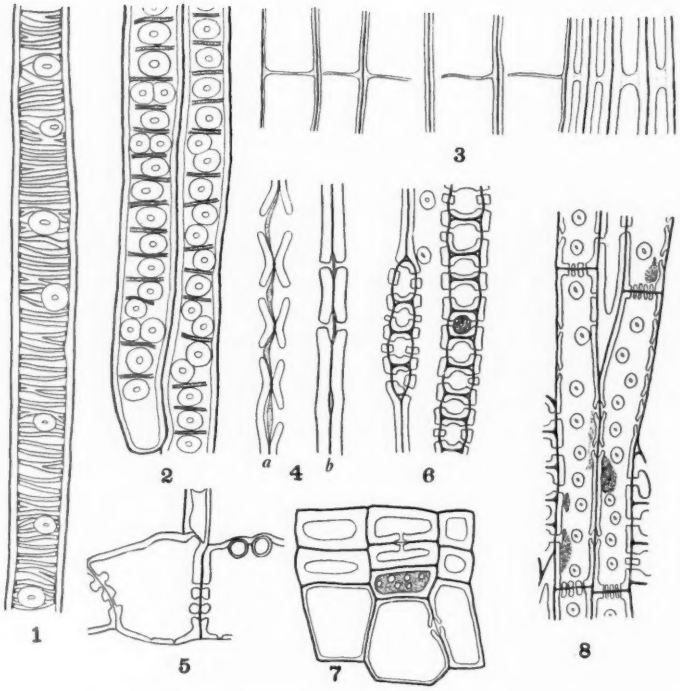
Introduction

So far as the writer is aware, the wood anatomy of *Pseudolarix* has been reported only once (3), and then rather superficially. JEFFREY, in his anatomical treatment of the Abietineae (9), bases his conclusions to a large extent on vascular anatomy and its phylogenetic significance. His main theses concern resin mechanism and ray tracheids. In works concerning the occurrence of single anatomical features in the Coniferales as a whole, GERRY (6) covers the distribution of the bars of Sanio, while ray tracheids are fully reported by HOLDEN (8). PENHALLOW (16) includes a more extensive anatomical study of six of the genera of Abietineae in his work on gymnosperms, although *Keteleeria*, *Cedrus*, and *Pseudolarix* are not considered. KANEHIRA (12, 13) in his comprehensive studies of Formosan and Japanese woods omits the genera *Cedrus* and *Pseudolarix*.

Along the lines of microscopic morphology, MIYAKE and YASUI (14) find the gametophytic development in *Pseudolarix* closely following the abietinean type. BUCHHOLZ (4) further observes the similarity in embryogeny of this genus to that of the Abietineae as a group.

The investigation reported here attempts to add to the knowledge of the wood anatomy of conifers as a whole and to supplement that of the already best known group, the Abietineae. The writer has undertaken to determine the many and diverse affinities that arise from microscopic anatomy and to integrate them into a logical phylogenetic relationship with other conifers.

The genus is monotypic and is indigenous to eastern China. The single species is known correctly as *Pseudolarix kaempferi* (Lindley) Gordon. A number of recent manuals include the morphological description of this genus, since it is cultivated at several plantings in the eastern United States.



FIGS. 1–8.—Fig. 1, closely spiraled metaxylem element showing rudimentary pits imbedded in spiral bands (radial). ×500. Fig. 2, bordered pits on tracheids of median spring wood, showing occasional biseriate arrangement (radial). ×240. Fig. 3, portion of trabecula passing from terminal wood to next year's growth (radial). ×240. Fig. 4, bordered pits in section contrasted: *a*, radial wall of spring wood; *b*, tangential wall of summer wood. ×500. Fig. 5, simple pit connection between marginal ray cell and final tracheid of summer wood (radial). ×500. Fig. 6, section through xylem rays in summer wood, showing connections with tracheid by means of simple pits (tangential). ×225. Fig. 7, end wall of wood parenchyma cell; note also bordered pit on tangential wall of late summer wood (transverse). ×225. Fig. 8, two entire wood parenchyma cells bordering rays and a tracheid by means of simple pits. Bordered pits connect these cells with each other and with following year's first spring tracheids; note also albuminous contents (tangential). ×225.

Investigation

General anatomical features

The vertical elements of *Pseudolarix* are wholly tracheidal, with the exception of terminal wood parenchyma. By this term is meant the occurrence of this cell type only in the last layer of summer wood, where it is abundant. The xylem rays are wholly parenchymatous.

The wood is white when cut and becomes darker on exposure. The heartwood becomes yellow-brown while the sapwood, which is 1–2 cm. in diameter, has a pale creamy shade. Specific gravity shows a figure of 0.534. The pith is approximately c.5–1 mm. in diameter, in the outer layers of which nuclei are evident in stems as much as nine years old.

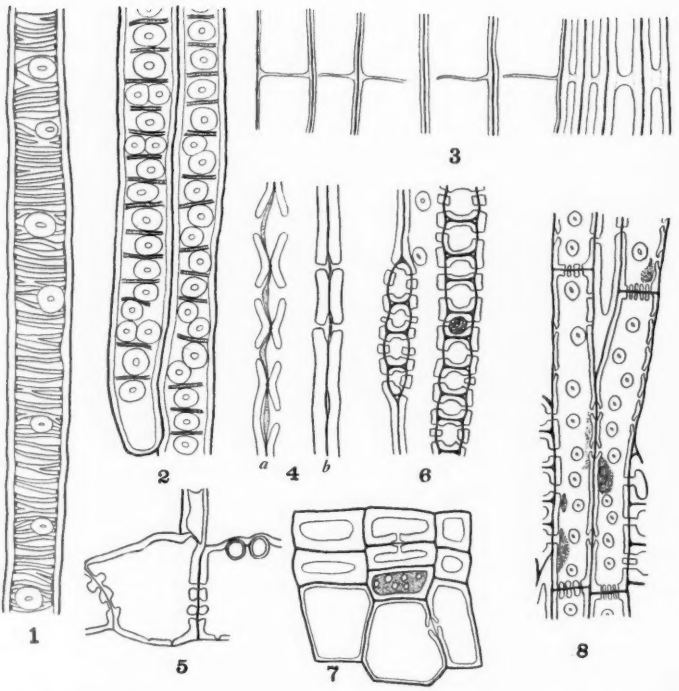
The transition from spring to summer wood is gradual, and shows no definite line of demarcation such as would be afforded by a girdle of resin cells or an abrupt thickening of tracheid walls. The extent of spring wood is from 20 to 30 cells, while that of summer wood is less, being from 8 to 18 cells. A distinct growth ring boundary is produced by the dense summer wood (fig. 9).

Stelar development is endarch, the condition typical of gymnosperms. The protoxylem is isolated, occurring at 15 to 20 points which project into the pith, and consists of thin-walled elements with spiral thickenings. The spirals are very loose in the first elements and become more compact, passing gradually into the type designated as metaxylem. These latter elements are closely spiraled, almost scalariform, and show an abundance of small rudimentary bordered pits imbedded in the bands (fig. 1). In the radial section passing through the greatest amount of protoxylem the extent is from three to five cells, while that of metaxylem is rather uniformly two or three cells.

Tracheids

Pseudolarix shows a moderate variation in tracheid length. The average is 2.5 mm., and a steady gradation includes the extremes of 1.8 and 3.4 mm. Table I gives the complete relative measurements of tracheidal parts.

Large and distinct bordered pits are uniformly abundant on radial walls, extending to the ends of cells (fig. 2). In uniseriate pitting the



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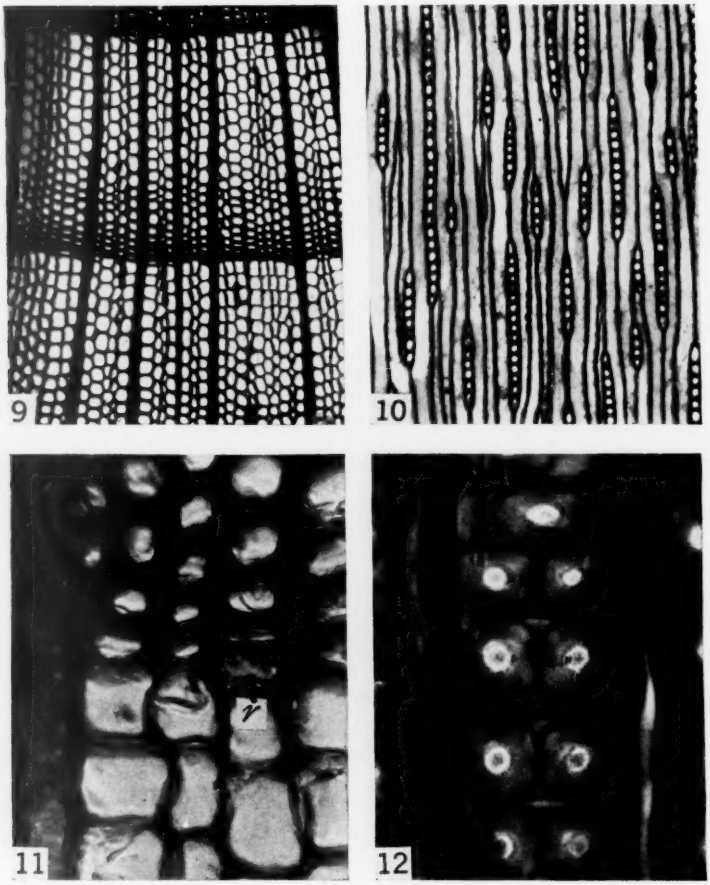
Large and distinct bordered pits are uniformly abundant on radial walls, extending to the ends of cells (fig. 2). In uniseriate pitting the



FIGS. 9–12.—Fig. 9, transverse section; note gradual transition yet distinct growth ring boundary. ×80. Fig. 10, tangential section; note height and straightness of uniformly uniseriate rays. ×80. Fig. 11, transverse section showing resin cell plate (*r*) in terminal summer wood. ×480. Fig. 12, radial section; note distinct horizontal crassulae and absence of vertical bars. ×800.

horizontal diameters are slightly greater than the vertical, while in the biseriate arrangement, featuring the first four to six layers of spring wood, the compressed pits become somewhat squared (figs. 12, 13). In both instances the pit mouth is about one-third the diameter of the entire pit. The pits of summer wood are smaller and the mouth takes on a characteristic slitlike appearance (fig. 14). Smaller bordered pits are also found in moderate abundance on tangential walls in the last two to four layers of summer wood (fig. 8). In cross-

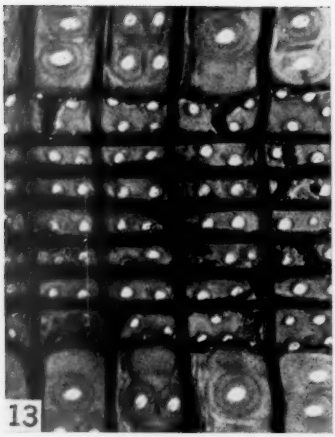
TABLE I

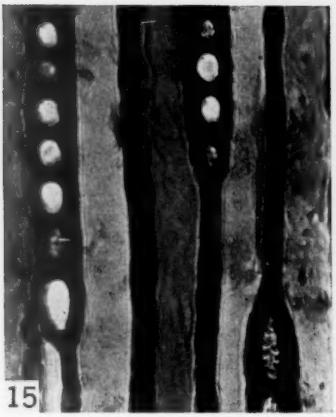
COMPARATIVE MEASUREMENTS OF PARTS OF TRACHEIDS, BASED ON FIFTY SAMPLES

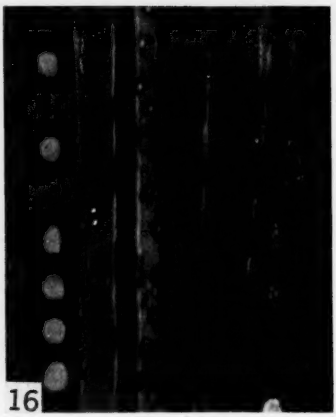
	SPRING WOOD (μ)	SUMMER WOOD (μ)
TRACHEID		
Length	2547	2547
Radial depth	39.0	11.7
Tangential width	35.1	30.2
Wall, radial	1.9	3.4
Wall, tangential	1.5	4.8
BORDERED PITS		
Radial walls, mouth, horizontal	7.0	4.1
Radial walls, mouth, vertical	4.4	4.1
Radial walls, entire, horizontal	21.4	13.8
Radial walls, entire, vertical	16.8	13.8
Tangential walls, mouth	.....	4.3
Tangential walls, entire	.....	13.9

section these show a decided contrast to the usual pits on radial walls of spring wood (figs. 4, 7). Cracking of pit mouths is observed in the summer wood tracheid, taking the form of right-hand striations which often form a continuous spiral in the final layers. Tracheids are in direct communication, through pits, with ray cells in all regions of the wood (figs. 13, 14). In the final two or three layers the connections are of simple pits (figs. 5, 6).

Crassulae (**19**) occur consistently throughout the spring wood and are of sporadic occurrence in the summer wood. They are chiefly single and straight, although variations are seen occasionally (fig. 13). An indication of vertical bars between pits of biseriate arrangement is afforded under low power magnification. However, the oil immersion objective proves these lines to be an illusion, probably of







FIGS. 13–16.—Fig. 13, radial section of spring wood showing characteristic tracheid pitting and lateral ray pits. ×280. Fig. 14, same, in summer wood region. Fig. 15, tangential section of spring wood showing radial pits in section, and detail of rays. ×300. Fig. 16, same of summer wood showing wood parenchyma cells and communication with ray cells; note also bordered pits on tangential walls of parenchyma and tracheids alike.

refraction, while the horizontal bars stand out conspicuously (figs. 12, 13).

Trabeculae (**15**) are sometimes present and extend through the growth of several years (fig. 3). These structures, apparently homogeneous with the wall of the element through which they pass, presumably arise at the time of the formation of each tracheid of the series by the cambium.

Hexagonal, and rarely rectangular, crystals have been occasionally observed in various positions in summer tracheids. They are usually longer than the tangential width of the element and cause a bulging when they lie horizontally.

Wood parenchyma

The wood parenchyma in *Pseudolarix* is strictly terminal, occupying almost the entire final layer of summer wood. The cells show a great variation in length, from 40 to 250 μ, and average roughly 150 μ. Their depth and width are fairly uniform, the former varying but slightly from 15 μ and the latter constant at 30–33 μ. These cells thus show a greater depth than summer tracheids, are approximately as wide, and are much shorter.

The longitudinal walls are slightly thinner than those of summer tracheids, while the invariably horizontal end walls are thick and coarse, marked with two to six or more simple pits (figs. 7, 8, 11, 16). Pitting on radial walls is variable; communication with ray cells is by means of simple pits, a feature striking in its clarity; bordered pits occur between adjacent parenchyma cells; while between parenchyma cell and tracheid the pits are simple (fig. 8). Bordered pits occur abundantly on the tangential walls (figs. 8, 16).

In stained sections wood parenchyma cells contain a granular substance, sometimes extensively distributed, sometimes drawn up in semblance of nuclei (fig. 8). The alkannin test indicates the presence of resin in sections prepared from dried material.

Xylem rays

Ray tracheids are not present in the normal wood of *Pseudolarix*, nor have any been detected in wounded tissues. Several facts combine to indicate that rays are, except in possibly unobserved in-

stances, wholly parenchymatous. These are: the presence of nuclei indiscriminately in both marginal and intervening cells; the similarity of cells in all regions of the ray; and the absence of dentations or ridges on transverse walls. Rays vary from one to twenty-eight cells in height, averaging between eight and nine, while the majority contain five to ten cells. They are without exception uniseriate and are straight tangentially (figs. 10, 15). Several instances of ray fusion have been observed, occurring more often as traumatic responses than as a normal condition. Such fusions are marked by great irregu-

TABLE II

MEASUREMENTS OF RAY CONSTITUENTS

	SPRING WOOD (μ)	SUMMER WOOD (μ)
End walls	2.2	3.0
Transverse walls	1.9	2.4
Radial walls	2.3	2.4
End wall pits	3.0	3.0
Transverse wall pits	4.0	3.5
Radial wall pits, mouth	5.9	3.0
Radial wall pits, entire	7.8	4.2

larities in cell shape. No splitting or divergence of rays already formed by fusion has been observed.

Wall structure of ray cells is uniform. The end walls are coarse, thick, and profusely marked with simple pits (fig. 13). Transverse walls are smooth, thinner, and broken sparingly by simple pits. Radial walls are also smooth and thin, with narrowly bordered "piciform" (2) pits occurring two to four to a crossfield in the spring wood and one to two in the summer wood, the number being governed apparently by the size of the crossfield (figs. 13, 14). Transverse walls, moreover, are straight in the spring wood but converge slightly at the ends in the summer wood, giving the cells a convex shape. End walls occur in a great variety of positions in spring wood, while in summer wood they are more consistently vertical.

Measurements of ray cells reveal a difference of length in spring and summer wood, while the height is constant, 21–25 μ. Length in spring wood gives the maximum figure of 200 μ and average figure

of 180 μ, while in summer wood the minimum is 120 μ and the average 140 μ. Marginal cells are slightly variable in height, the distal wall showing gradual undulation. Table II gives the measurements of ray cell walls and pits.

Discussion

The type of tracheid pitting, in its clarity and abundance, shows similarity to the Taxodineae in general and to the majority of the Abietineae, with the exception of the few species of *Pinus* which lack this feature altogether (**13**). The form and distribution of crassulae show strong affinity to both the Abietineae and the Taxodineae (**6**).

The absence of resin passages constitutes a condition typical of JEFFREY'S subtribe Abieteae (**9**), one genus of which, *Abies*, may be designated as transitory since these structures occur in at least three of its species: *A. nobilis* (**16**), *A. firma* (**13**), and *A. venusta* (**18**). Fusiform rays, rays containing horizontal resin passages, are produced traumatically in *Cedrus* (**11**), the accepted primitive genus of Abieteae. These structures are not reported for any of the other genera, including the material of *Pseudolarix* observed by the writer.

The terminal distribution of wood parenchyma in *Pseudolarix* is duplicated, in the Abieteae, by *Tsuga* (**16**) and *Cedrus* (**11**), and in the Pineae by *Picea*, *Pseudotsuga*, and *Larix* (**11**). However, JEFFREY further asserts that *Tsuga* has a tendency toward diffuse distribution. In the matter of wood parenchyma distribution, therefore, *Pseudolarix* is grouped with *Cedrus* as showing the primitive type. In the entire Taxodineae the distribution of wood parenchyma is either diffuse or girdled in early summer wood, but never strictly terminal.

Pseudolarix is the only genus of the Abietineae without ray tracheids, and thus is similar to the Taxodineae, where they are rarely found (**10, 7**). The writer agrees with HOLDEN, who was unable to find ray tracheids, even in severely wounded branches. This genus appears to be little affected by wounding, however, as HOLDEN states further that the formation of traumatic resin passages is much less marked here than in other genera of the Abietineae. The fact that the writer also was unable to find these structures as a traumatic response serves to uphold this statement.

Ray cell pitting shows a great similarity to the Abietineae with the exception of most species of *Pinus* (**1, 2, 5, 11**). Moreover, the shape and occurrence of these pits strongly suggest the type found in *Sequoia* (**7, 16**) and *Cunninghamia* (**10, 13**), but the wide variation shown in the remaining genera of the Taxodineae is not present in *Pseudolarix*. BAILEY (**2**) reports great variation in ray lateral pitting in the hard and soft pines alike, and regards the piciform type as primitive. The nut pines exhibit the latter type and compare favorably with Cretaceous specimens. Since the majority of the Abietineae, however, and even of conifers in general, have distinctly piciform pits, we may confine the evolutionary sequence there described to the genus *Pinus*. As the ray structure of *Cedrus* stands midway between the Pineae and the Abieteae (**5**), so does that of *Pseudolarix* appear to be transitional between the Abietineae and the Taxodineae.

The spiral sculpture observed in summer wood tracheids of *Pseudolarix* is similar to that reported for *Pinus*, *Picea*, *Larix*, and *Pseudotsuga* (**1**), and *Keteleeria* and *Tsuga* (**12**). This feature is almost entirely foreign to the tribes Taxodineae and Cupressineae (**13**), and the spirals of *Taxus* and *Torreya* are easily recognizable as structurally different. Spirals therefore serve to unite the genera of the Abietineae without apparently exhibiting an evolutionary sequence.

Summary

The anatomical characters reveal that *Pseudolarix* occupies a relatively high position in the Abietineae. The anatomy, moreover, merges with that of the Taxodineae in a number of features, indicating a general transition in this direction.

The writer is indebted to Dr. JOHN T. BUCHHOLZ for furnishing the material, collected by him on Long Island, New York, and for his valuable suggestions and criticisms of the work.

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ELECTROPHORESIS OF LATEX AND CHROMOSOME NUMBERS OF POINSETTIAS

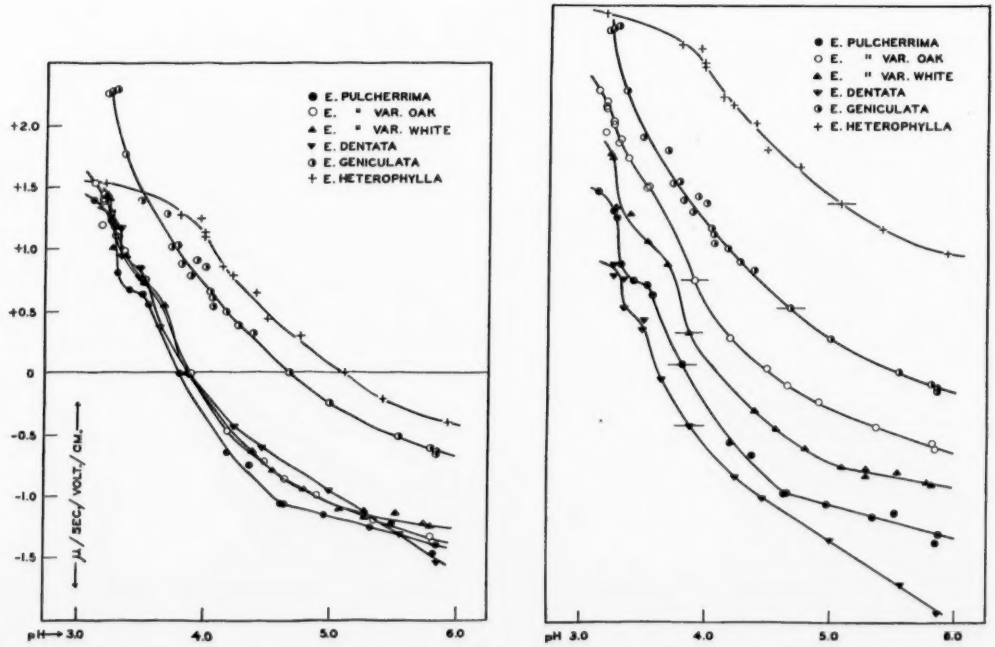
LAURENCE S. MOYER

(WITH NINE FIGURES)

In a recent paper (**10**) the writer presented determinations of the isoelectric points of the latex particles from more than twenty species of *Euphorbia*. Each latex was suspended in a series of M/50 acetate buffers and its electrophoretic mobility determined in a modified Northrup-Kunitz microelectrophoresis apparatus. The curves of mobility (in μ/sec./volt./cm.) plotted against pH proved to be constant for each of the species investigated. These curves (including isoelectric points) can be arranged into several different groups whose members have similar shapes. With few exceptions the groups agree with the taxonomic relationships already established for the genus.

The most noticeable exception occurred in the section Poinsettia. In this subdivision all of the investigated species are taxonomically very closely related (**3**). As may be seen in figure 1, *Euphorbia dentata* Mich. and *E. pulcherrima* Willd., with its two varietal forms, white and oak, are very similar in respect to shape of curve and isoelectric points (i.p.) In figure 2 the curves are plotted on the same scale but spread apart to show this similarity in shape. *E. heterophylla* L., another member of the group, however, shows no close relationship to the other species in either isoelectric point or in curve shape.

The writer has recently investigated the electrophoretic behavior of the latex from *E. geniculata* Orteg., another member of the section, in addition to the species whose curves are shown. This species was grown from seed generously supplied by the Muséum National d'Histoire Naturelle, Paris. A detailed description of methods employed is given in an earlier paper (**10**). The curve of mobility has been drawn to the same scale as the other curves, and is included in figures 1 and 2. As in the previous work (**10**), all points are corrected for temperature and are calculated to 25° C. The latex was tapped



FIGS. 1–2.—Fig. 1, mobility curves of latex particles from the section Poinsettia; all species plotted to same scale. In fig. 2 (on right), curves are spread out to show shapes.

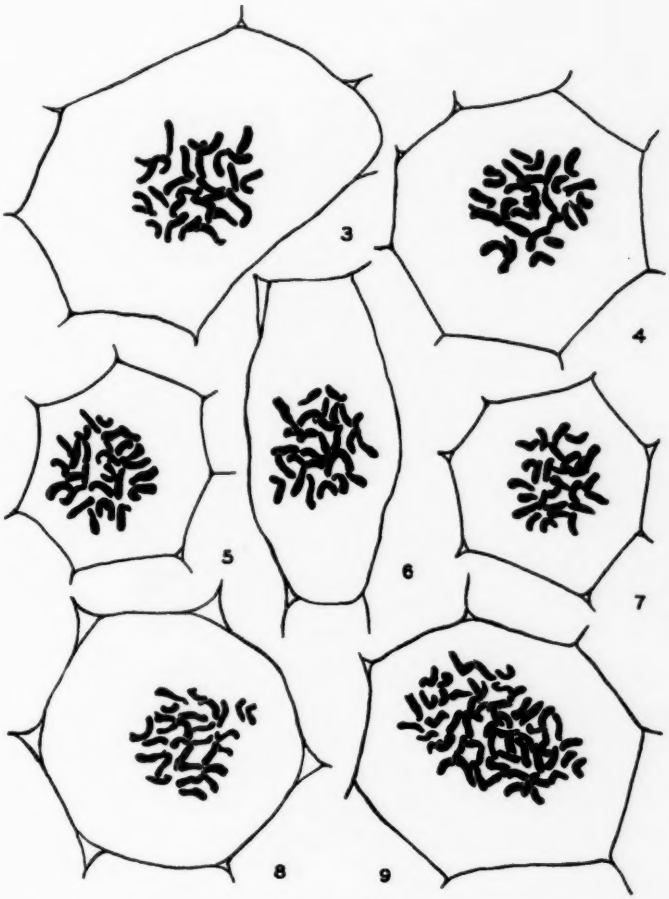
from four healthy specimens in order to eliminate individual variations as much as possible.

The curve is smooth and most like that of *E. pulcherrima* var. oak. The isoelectric points of the two curves, however, are widely divergent. The latex particles of *E. geniculata* do not move in an electric field at pH 4.7, while those from *E. pulcherrima* var. oak are isoelectric at pH 3.9. The curve for *E. heterophylla* is no more closely related by its shape to *E. geniculata* than to the members of the *E. dentata-E. pulcherrima* group.

Since the position of the point at pH 3.2 on the *E. heterophylla* curve caused most of the difference in shape between it and the curve for *E. geniculata*, this point was checked again and found to have the same position (within experimental error) as reported previously (**10**). The i.p. of particles of *E. heterophylla* latex was redetermined with plants grown from different seed and was again found at pH 5.1. This is not very close to that of *E. geniculata*. Since neither the systematic position nor the geographical distribution of these species offers a clear explanation for this divergence, it was thought that an investigation of chromosome numbers might be of value.

CARANO (**4**) reported that *Poinsettia pulcherrima* R. Grah. (=*E. pulcherrima* Willd.) has 10 chromosomes in the haploid cells "con sufficiente approssimazione." The chromosomes of the other species and varieties in this section apparently have never been counted.

Root tips were fixed in Nawaschin's fluid as modified by SAX (**14**) and dehydrated and imbedded in paraffin by means of ZIRKLE'S n-butyl alcohol technique (**17**). Transverse sections were cut 10μ thick, stained in crystal violet, and destained by NEWTON'S iodine method (**11**). A 15× orthoscopic ocular, a Zeiss HI 100 objective, n.a. 1.30, and a camera lucida were used in counting and drawing. ZETTNOW'S solution of copper sulphate and potassium bichromate diluted to one-third was used as a light filter. This solution gives nearly monochromatic light and makes objects stained violet appear as black. The results of the counts are given in table I while figures 3–9 show drawings of typical somatic metaphase plates. The table also presents data for a hybrid, *E. pulcherrima* var. oak ×*E. pulcherrima* var. white, obtained from the Missouri Botanical Gar-



FIGS. 3–9.—Somatic metaphase plates of poinsettias: fig. 3, *Euphorbia pulcherrima* var. white, 2n = 28; fig. 4, *E. pulcherrima* var. oak, 2n = 28; fig. 5, *E. pulcherrima* var. oak × white, 2n = 28; fig. 6, *E. pulcherrima*, 2n = 28; fig. 7, *E. geniculata*, 2n = 28; fig. 8, *E. dentata*, 2n = 28; fig. 9, *E. heterophylla*, 2n = 56. × 2000.

den (2). This hybrid has pink bracts and leaves similar to the oak variety. No electrophoretic curve is given for this cross.

All of the species investigated in this group which have similar surfaces on their latex particles, as shown by the resemblance of the curves and the position of the isoelectric points (table I), have also like chromosome numbers. Although the curve of *E. geniculata* is somewhat divergent, it likewise has a diploid number of 28. On the other hand, *E. heterophylla* appears to be tetraploid with a somatic complement of 56 chromosomes, and it is therefore genetically very different from the other species.

TABLE I

CHROMOSOME NUMBERS AND ISOELECTRIC POINTS OF POINSETTIAS

SPECIES	LATEX ISOELECTRIC POINT (pH)	CHROMOSOME NUMBER (2N)
E. dentata	3.9	28
E. pulcherrima	3.8	28
E. pulcherrima var. white	3.9	28
E. pulcherrima var. oak	3.9	28
E. pulcherrima var. white×oak	3.7	28
E. geniculata	4.7	28
E. heterophylla	5.1	56

In this connection it is interesting to note that ZADE, by the immunological reactions of the seed proteins (16), was able to separate the various species of *Triticum* and *Avena* into groups which are identical with those already formulated on a taxonomic basis. In a study of the chromosomes of *Triticum*, SAKAMURA (12) and later SAX (13) reported that each group has different chromosome numbers. The haploid numbers are as follows: einkorn group = 7, emmer group = 14, and spelt group = 21. KIHARA (7) likewise showed the same type of relationship between the chromosome numbers of different species of *Avena* and the groups proposed by ZADE. In a chemical study of the purified prolamines of corns and wheats, HOFFMAN and GORTNER (6) were unable to show any essential differences between the prolamines from the three wheat groups but were able to differentiate the wheat and corn groups. Their work was

confirmed by LEWIS and WELLS (**8**), who investigated the anaphylactic reactions of these proteins. Hence it is obvious that the mixed seed proteins (which presumably included the nucleoproteins) of wheat show differences which correspond to natural relationships and which are not shown by the purified prolamines.

Chromosome numbers of different members of the Centrospermae have also been shown by TISCHLER (**15**) to be correlated with the taxonomic groups proposed by MEZ and ZIEGENSPECK (**9**) on the basis of their serological reactions.

FUKUSHIMA and MARUYAMA (**5**) have recently investigated the immunological relationships and chromosome numbers of eight species of *Brassica*, and they find that the species fall into four groups. The members of each group have the same chromosome number and protein relationships, yet they are noticeably different from the members of any of the other three groups.

In the case of the latex particles of poinsettias, which are coated at least in part with proteins, the electrophoretic differences may not be due to a specific difference in the chemical constitution of the proteins themselves but to a difference in the proportions of the several proteins adsorbed on the surfaces. The composition of this hypothetical mixture appears to be constant in each species and to differ little among individual plants, but the proportions are evidently different in different species. When the curves are arranged in order of decreasing i.p., as noted before, they become less smooth as we pass down the pH scale (fig. 2), until the smooth even curve of *E. geniculata* is reached. The shape of this curve is almost identical with those of the curves obtained for pure proteins (**1**). Possibly the curves of the other members of the 28-chromosome group are conditioned by a mixture on the surfaces of the particles which is composed of protein in decreasing amounts plus another ampholyte of low i.p. If more species were investigated, a complete series might be found extending from *E. dentata* to *E. geniculata*. It should be noted, however, that the isoelectric points when plotted on the usual pH scale show much less divergence than when the actual concentrations of hydrogen ions are expressed. The difference between the i.p. of *E. pulcherrima* and that of *E. geniculata* is then ten times the difference between that of *E. geniculata* and *E. heterophylla*.

Both the electrophoretic behavior of these latices and their chromosome numbers appear to divide the species into three groups. The first group, composed of *E. pulcherrima* with its varieties and *E. dentata*, is linked to some extent to *E. geniculata* by an identical chromosome number (not necessarily an indicator of close relationship, to be sure) and possibly by its curve shape, but it differs in the position of the i.p. *E. heterophylla*, with 56 chromosomes and with both a different curve shape and a different i.p., does not seem clearly linked to either of the other groups.

Summary

1. The electrophoretic mobility curve for the latex particles of *Euphorbia geniculata* Orteg. has been determined in a modified Northrup-Kunitz apparatus.

2. The curve of *E. geniculata* latex is definitely different in shape and in the position of the isoelectric point from *E. heterophylla*, but, although its isoelectric point differs from those of *E. dentata* and of *E. pulcherrima* and its varieties (which lie close together), it seems related to them in shape.

3. The chromosomes from the root tip cells of four species and three varieties of the genus *Euphorbia*, section Poinsettia, have been counted. *E. dentata* Mich., *E. pulcherrima* Willd., *E. pulcherrima* var. oak, *E. pulcherrima* var. white, *E. pulcherrima* var. white × *E. pulcherrima* var. oak, and *E. geniculata* Orteg. are all characterized by a diploid chromosome number of 28, while *E. heterophylla* L. is tetraploid with a somatic number of 56.

4. These chromosome numbers are correlated with the grouping determined by the electrophoretic mobility curves and isoelectric points of latex particles found for the section.

The writer is indebted to Professor CONWAY ZIRKLE for valuable advice and assistance.

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ORIGIN OF ADVENTITIOUS ROOTS IN COTONEASTER DAMMERI

FLORENCE WOLFE

(WITH NINE FIGURES)

Introduction

In general, the term adventitious refers to any organ produced on the body of a plant in an abnormal position. Shoots produced from roots, or roots from aerial stems and leaves, are examples of this type of organ. Aerial stems of many species of plants show a tendency to produce adventitious roots, especially when they come in close contact with the soil. These roots may appear at any place on the stem, or in special positions, such as in the axils of buds or below the buds. Their origin is dependent upon the continued organized activity of one or more meristematic cells, or upon the renewal of meristematic activity of some other cell or group of cells. The present study was made to determine the origin of the adventitious roots on the stem of *Cotoneaster dammeri* Schneid. (*C. humifusa* Duthie) (1).

A review of the literature indicates that most of the investigation dealing with adventitious roots on stems is comparatively recent. CORBETT (4), in working with both woody and herbaceous cuttings, states that "roots take their origin in and are an extension of the active, formative material of the cambium." He maintains that the place of origin in cuttings of hardwoods is nearer the true cambium than it is in herbaceous cuttings of *Geranium*, *Coleus*, and allied plants. VAN DER LEK (5) states that roots of cuttings of *Salix*, *Populus*, and *Ribes nigrum* originate from preformed "root germs." These he reports to be closely associated with the cambium and located at the end of a medullary ray. WILSON (11) finds that epidermal and cortical cells in the axil of a branch bud of *Roripa austriaca* divide and give rise to the new root. SMITH'S investigations (8) show that the root initial in cuttings of *Clematis* are derived from the fascicular cambium, and that the earliest embryonic stages extend from the cambium layer into a medullary ray. TAYLOR (9), in his

experiments with cuttings of *Acanthus montanus*, concludes that the meristems of roots originate from the cambium. PRIESTLEY and SWINGLE (7) find that roots which arise upon the young stems of *Veronica beccabunga* are pericyclic in position. Furthermore, they believe that the roots are not derived from one cell, but "are a result of the organization of a group of cells bordering upon the vascular cambium which have remained or again become meristematic." LEMAIRE (6) and VAN TIEGHEM and DOULIOT (10) contend that root primordia of adventitious roots on stems originate from the pericycle. CONNARD and ZIMMERMAN (3) find that adventitious roots from cuttings of *Portulaca oleracea* arise in the medullary rays from the interfascicular cambium. CARLSON (2), in work on *Coleus blumei*, states that "adventitious roots arising between the vascular bundles from the bases of young cuttings originate in one to several adjacent cells of the pericycle."

With the exception of the one case just mentioned in which the adventitious roots originated from the cortical and epidermal cells, all previous research indicates that these roots originate from cells of either the cambium or pericycle.

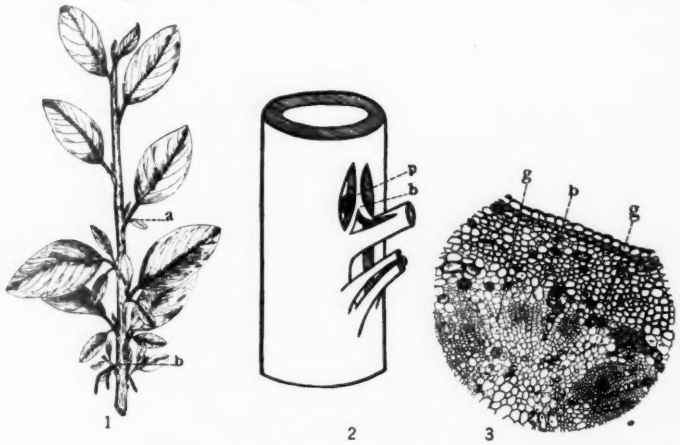
There are about forty species of *Cotoneaster* growing in the temperate regions of Europe, Asia, and North Africa. They are woody plants of the family Rosaceae, subfamily Pomeae. *Cotoneaster dammeri*, or *C. humifusa* as it is sometimes called, was introduced into the United States a quarter of a century ago from the mountains of China. It is an evergreen prostrate form, and is grown chiefly for landscaping purposes because of its showy fruits (12). As it creeps over the ground, it readily gives rise to a single root in the axil of each bud.

Investigation

Several plants of this species were obtained in September from a nursery and placed in a greenhouse for the winter. During the fall and early winter months, sections of the stem about 1 cm. in length were cut from the nodes, fixed in formalin-acetic-alcohol, and imbedded in paraffin. Cross and longitudinal sections, 10 μ in thickness, were made, mounted in series on slides, and stained with safranin and gentian violet.

At first a single small protuberance appears in the axil of each

bud, beginning at the third to sixth nodes from the tip of the growing stem (fig. 1*a*). The protuberances become larger and larger in descending order to the tenth to fifteenth nodes, where fully formed roots are present. They emerge perpendicularly to the long axis of the stem at one side of the protuberance. The long axis of each root forms a 30° angle, with a radial line drawn from the center of the stem through the center of the long axis of the bud. The fact that these roots appear at such regular positions leads one to conclude



FIGS. 1–3.—Fig. 1, sketch of twig showing consecutive development of protuberances and roots in bud axils (*a*, protuberance; *b*, fully developed root after emergence, showing tendency to branch). Fig. 2, diagram of stele showing three leaf traces, two branch traces, leaf gap, divided bud gap, and position of root primordium (*b*, vascular tissue of divided bud gap; *p*, position of root primordium). Fig. 3, portion of cross-section near tip of stem taken through bud gap, showing epidermis, connection of cortex with gap and pith parenchyma, vascular tissue of stem on either side of gap, and strands of undifferentiated vascular tissue in gap (*b*, strands of immature vascular tissue; *g*, parenchyma of gap).

that a cell or a group of cells in a specific position gives rise to the root primordium.

The structure of *Cotoneaster dammeri* does not differ greatly from that of most woody plants. The very young stem shows a dictyostelic arrangement of the vascular tissues with collateral bundles. However, this is not the case in older portions of the twig. In sections beginning with the fourth node from the tip the vascular

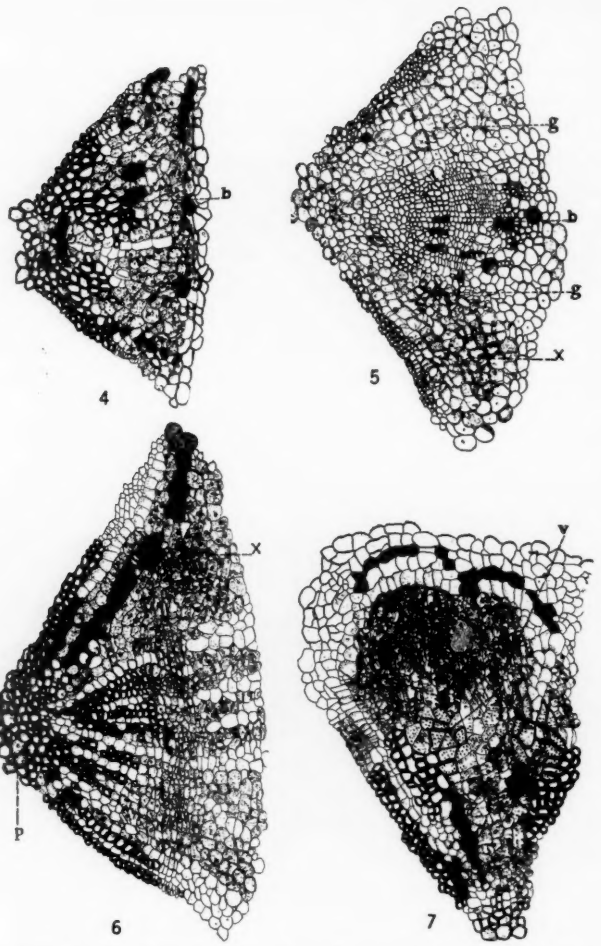
strands are connected laterally by the lateral extensions of the first formed ones, thus forming a continuous cylinder. Three vascular bundles arising from the stele supply the leaves and constitute the leaf trace. These bundles pass out into the petiole of the leaf at a sharp angle from the stem and unite very early to form a single bundle within the petiole. Figure 2 shows the stele with leaf trace.

The leaf gap is narrow and extends but a short distance above the point where the traces leave the vascular cylinder. This gap does not close, but merges with the branch gap, as will be shown later.

Two vascular bundles, the branch traces, arise from the vascular ring on either side of the leaf gap and unite within a short distance to form a complete stele in the young branch. The gap left in the vascular ring of the stem by the outward growth of these branch traces is continuous with that of the leaf gap (fig. 2), although it is slightly greater in width. The leaf and branch gaps are both composed of parenchyma and extend from the pith to the cells of the cortex.

The branch gap extends longitudinally to a distance equal to the length of a fully formed bud. It increases in width for a short distance and then gradually decreases until it becomes entirely closed. Generally a few strands of vascular tissue arise from the branch traces as they approach the outer portion of the stem (fig. 2*b*). These strands pass up through the branch gap and in most cases unite to form a small irregular bundle in the upper central portion (figs. 2*b*, 3*b*, 4*b*, 5*b*). Figures 4, 5, and 6 are portions of a cross-section of the stem at the branch gap through the median line of development of the root primordium, and include only the tissues of the branch gap extending from the pith to the cortex, with a small number of cells of the vascular tissue of the stele on either side. The maturation of the vascular tissue in the gap is not completed so soon as that of the vascular ring (fig. 3*b*). This last section is one taken from a branch gap near the tip of the stem and includes the cortex and epidermis beyond the branch gap.

Sometimes the union of the scattered strands does not always occur (fig. 3*b*). In most cases, however, the parenchyma of the gap becomes divided into two parts, occupying a position on either side of the central strand of vascular tissue (fig. 5*g*). The cells of this parenchyma are thin walled and have large vacuoles.



FIGS. 4–7.—Portions of cross-sections through successively older nodes at median line of development of root primordia. Fig. 4, very early stage in formation of root primordium with two vascular strands in gap. Fig. 5, older stage of root development showing recent divisions of cells with single vascular strand in center of gap. No development of cells can be detected on other side of gap in root formation (*g*, parenchyma of gap; *b*, vascular tissue; *x*, early development of root primordium). Fig. 6, later stage of primordial development than in fig. 5, showing elongation of cells between center of meristematic activity and vascular tissue (*x*, elongating cells at base of meristematic group). Fig. 7, one half of gap on side of root development, showing (left) still older root primordium with tracheids connecting with vascular cylinder of stem and (right) vascular tissue of gap (*v*, tracheids of root connecting with vascular strands of stem).

The outer layers of pith, when mature, consist of small cells that are much thicker walled than those of the branch gap (fig. 6*p*).

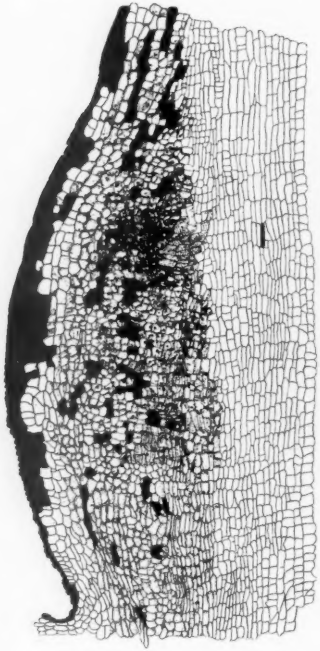
The epidermal cells become heavily cutinized, and the outer cells of the cortex develop into chlorenchyma at an early stage. No endodermis is present, as is generally the case in most woody stems of angiosperms. The pericycle varies in thickness from three to five cells, and in many cases is very difficult to distinguish as it merges into the thin walled cells of the inner cortex. In older, well developed stems, pericyclic fibers are prominent in scattered groups around the outside of the primary phloem (fig. 9*f*). This figure is a section of the stem through a fully developed root. The amount and development of secondary xylem and phloem depend entirely upon the age of the portion of the stem in question, and need no further discussion at this time. Scattered cells in various parts of the stem are filled with tannin and appear very dark after staining.

For the study of very early stages, serial sections were examined from the first three nodes at the top of the stem. The parenchymatous tissue of the gap was well divided into two groups, one on either side of the group of immature vascular tissue (fig. 3*g*). None of the cells in the divided gap revealed any indication of meristematic activity. The thin walled cells with their inconspicuous nuclei gave evidence that no unusual activity was taking place.

Figure 4 represents a section similar to the preceding one, but made from a slightly older node. Cell division is evident on one side of the gap near the cortex, owing to the appearance of abundant cytoplasmic contents and large nuclei in the cells. The activity of these cells is different from that of a similar group on the opposite side of the gap, or from those in a like position in figure 3.

In sections of slightly older nodes the meristematic region increases in size (fig. 5*x*), owing to the division of the original cells of the group and the reactivation of surrounding parenchyma cells. In figure 5 approximately 15 cells in one plane are involved, with evidence of at least two very recent divisions near the center of the group. In older nodes greater activity is apparent (fig. 6*x*). Many of the cells near the center of the active group show recent division, occupying about the same amount of space as the mother cells from which they arose. At the base of the group some of the cells have elongated.

These later develop into vascular tissue. Figure 7 shows the mass of active tissue becoming rounded, with the elongated cells at the base developing into vascular tissue which connects with the vascular elements of the stem on one side and with that of the center of the gap on the other.



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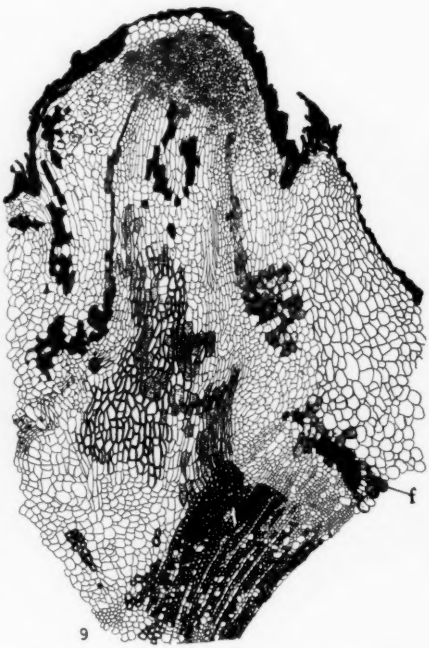

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FIGS. 8, 9.—Fig. 8, longitudinal section through protuberance, showing primordium in approximately same stage as in fig. 6. Cells at base of root show differentiation into tracheids; cells of cortex show presence of tannin in darkly stained areas, and at lower left a portion of upper part of bud is shown. Fig. 9, fully differentiated root breaking through cortex and epidermis with vascular tissue connecting with vascular cylinder of stem.

The structure of the root primordium and its position relative to the gap may be better understood from a longitudinal section cut in direct line, with the parenchyma on one side of the bud gap because of the vertical orientation of the parts. Figure 8 illustrates such a

section in about the same stage of development as in figure 7. The small projection at the lower left shows the point of attachment of the upper portion of the bud. The cortex is continuous with the parenchyma of the gap and finally merges into the central pith region at the right. The formation of the protuberance out of which the young root emerges is due, not only to the formation of the root primordium, but also to the division of some of the cells in the cortex. As shown in this section the root originates slightly above the center of the protuberance. The black body in figure 2 illustrates its position with reference to the stele.

From this time on development is rapid if conditions are favorable, that is, if the stem is in close contact with the soil. If such is not the case the root remains in a state of dormancy inside the protuberance until conditions are favorable for renewal of activity. Differentiation into the various root tissues occurs as the root forces its way outward. It increases rapidly in diameter, while the cells back of the tip elongate and form the cortex and central cylinder. The connection of the vascular tissue of the root and stem is completed (fig. 9) and the endodermis becomes clearly distinguished. The root apparently forces its way through the cortex and epidermis by pressure, although some chemical dissolution may take place. In many cases the root branches after attaining a length of half an inch (fig. 1*b*).

The unusual feature in the entire development is that the root primordium originates on one side of the gap only. Although the parenchyma tissue on both sides of the branch gap appears identical in the early growth of the stem, no development occurs except on one side. This is due to resumption of the activity of these cells.

Summary

1. Adventitious roots of *Cotoneaster dammeri* arise from only one of the two groups of parenchymatous cells in the divided bud gap.

2. Formation of the root primordium is apparently due to the resumption of activity of the parenchyma cells.

3. The cortical cells in the region of the bud axis produce a slight protuberance through which the young root emerges.

4. Vascular elements of the young root arise very early.

5. The root is fully differentiated into the cortex and central cylinder by the time that it is ready to emerge.

6. Development of root initials occurs consecutively as the stem elongates.

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NEW OR INTERESTING MYXOPHYCEAE FROM MISSOURI

FRANCIS DROUET

(WITH TWELVE FIGURES)

A few new or otherwise interesting blue-green algae have appeared in my collections from time to time during the past five years. The recent discovery of a number of unidentified specimens in the Herbarium of the Missouri Botanical Garden, collected by Dr. WILLIAM TRELEASE, brought to light several species in this group which have not previously appeared in the lists of Missouri algae (**2, 3, 5, 6**). *Scytonema alatum* (Carm.) Borzi has lately been recorded from Shannon Co. by SHARP (**8**).

The Myxophyceae are a difficult group, not only because of their few and minute taxonomic characters, but also because these characters may be easily changed by the mode of preservation. Formalin and other fixing agents produce granules and distortions of shape not found in living material. Dried algae are not always satisfactory for study. Living material cannot always be conveniently examined, especially on collecting trips of more than a day's duration, since deterioration sets in shortly after collection. The most satisfactory method of fixing which I have found is with a 3–5% aqueous solution of commercial formalin (40% formaldehyde). Plenty of liquid should be used so that the killing agent may penetrate throughout the mass as quickly as possible. Most of the observations on fixed material have been supplemented by the examination of living material.

BOYE PETERSEN (**1**) and GEITLER (**4**) have discussed the relative merits of diagnostic characters among the blue-green algae, and I have here placed most emphasis upon size and shape of cells, especially those at the ends of the trichomes, and the reaction of chlor-zinc-iodine upon the sheaths. Chlor-zinc-iodine[1] solution is prepared

[1] The latin equivalent of the name of the reagent, chlor-zinc-iodine, as employed by GOMONT, is *chlorozincicum ioduratum*, which, when translated, means "iodinized chlor-zinc."

according to the formula given by NOWOPOKROWSKY (7), here translated as follows:

> Dissolve 20 gm. of $ZnCl_2$ in 8.5 cc. of water with the aid of heat. Cool and add, drop by drop, the following solution until, after shaking, the iodine which is precipitated fails to dissolve further: 3 gm. of KI, 1.5 gm. of I_2, and 60 cc. of water. About 1.5 cc. of the latter solution is necessary.

MYXOPHYCEAE

Chroococcaceae

MICROCYSTIS FLOS-AQUAE (Wittr.) Kirchner *in* ENGLER-PRANTL, Natür. Pflanzenfam. **1(1a)**:56. 1900.

Cells 2–7 μ diam. Fig. 1.

Floating in pond near Brandsville, Howell Co., Aug. 21, 1928, *Drouet 176*.

Oscillatoriaceae

PHORMIDIUM CORIUM Gomont, Monogr. Oscill. 172. 1892.

Trichomes 3–5.6 μ diam. Type measures 3–4.5 μ diam. Sheaths violet when treated with chlor-zinc-iodine. Fig. 2.

"Stone-Lawrence Co. line," Sept. 10, 1898, *Wm. Trelease*, in Herb. Mo. Bot. Gard.

PHORMIDIUM PURPURASCENS (Kütz.) Gomont var. **elegans,** var. nov.

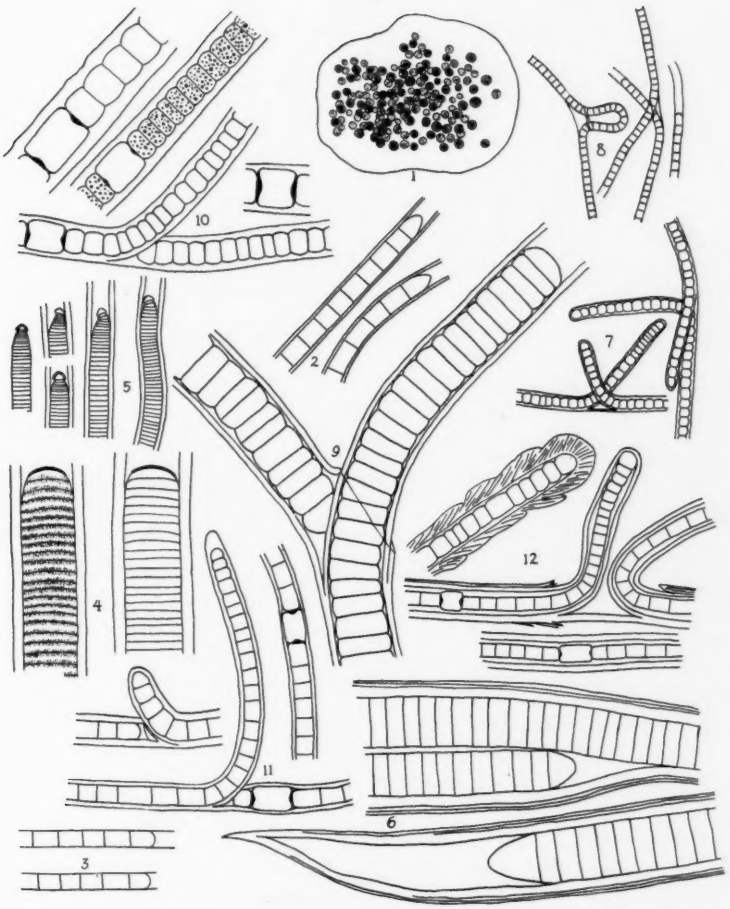
Var. cum trichomatibus 3–5.5 μ latis. Hab. in aqua artesia, Missouri.

Like the type, but with trichomes 3–3.5 μ diam. Fig. 3.

This alga formed violet-black leathery sheets on débris and water plants in a shady spring beside Highway 28 at the Gasconade River in Pulaski Co. The form answers exactly to the description of *Ph. purpurascens* (Kütz.) Gomont except for the larger size of the trichomes. The sheaths do not stain violet with chlor-zinc-iodine. Oct. 1, 1932, *Drouet 1030, 1033*.

LYNGBYA AESTUARII (Mertens) Liebm. forma *symplocoides* Gomont, *in* GEITLER, Cyanophyceae (Blaualgen). 1053. 1932.

Trichomes mostly 13–16.5 μ wide. Sheaths colorless with chlor-zinc-iodine. Fig. 4.



FIGS. 1–12.—Fig. 1, *Microcystis flos-aquae*, one colony showing gas vacuoles in cells. Fig. 2, *Phormidium corium*, ends of filaments. Fig. 3, *Ph. purpurascens* var. *elegans*, ends of filaments. Fig. 4, *Lyngbya aestuarii*. Fig. 5, *L. hahatonkensis*, showing variations of apices of trichomes. Fig. 6, *Hydrocoleus ravenellii*, showing typical end cell and presence of two trichomes within a sheath. Fig. 7, *Plectonema boryanum*, showing duplicate branching. Fig. 8, *P. notatum* var. *saegeri*. Fig. 9, *P. tomasinianum* var. *gracile*. Fig. 10, *Scytonema austinii*, showing variation in size of trichomes and habit of branching. Fig. 11, *S. hofmanni*. Fig. 12, *S. mirabile*. All ×600.

Forming *Symploca*-like mats on mud in a marsh fed by salt springs, Chouteau Springs, Cooper Co., May 3, Oct. 25, 1931, *Drouet 879, 933, 936*.

Lyngbya hahatonkensis sp. nov.

Strato nigrovirescente et laminoso; filamentis 6–7.5 μ diam., saepe plus minus curvatis et intricatis; vaginis chlorozincico iodurato non caerulescentibus, hyalinis, firmis, 1–2.5 μ latis; trichomatibus caeruleis, non constrictis, auguste attenuatis et ad apices vix curvatis, distincte capitatis; cellulis 4–5.2 μ crassis, brevissimis, 0.5–1.5 μ longis; cellula apicale conica, calyptram portante. Hab. in aqua artesia, Hahatonka, Missouri.

Filaments aggregated into dark blue-green strata, 6–7.5 μ in diameter, rather long, usually curved and entangled; sheaths well defined, not colored violet with chlor-zinc-iodine, hyaline, not laminated, sometimes roughened in age, 1–2.5 μ wide; trichomes bright blue-green (in living material), not constricted at cross walls, evidently narrowed at ends and slightly bent or spiraled, usually distinctly capitate; cells 4–5.2 μ in diameter, very short, 0.5–1.5 μ long; end cell conical, with conspicuous calyptra. Fig. 5.

In the drip of an artesian spring at Hahatonka, Camden Co., Aug. 9, 1928, *Drouet 148*.

The walls are usually somewhat granulated, although this is not always the case. Many granules appear throughout the protoplasm after the trichomes have been immersed in aqueous formalin. The form suggests *L. martensiana* var. *minor* Gardner, but in *L. hahatonkensis* the cells are much shorter and the ends of the trichomes more distinctly capitate (and calyptrate) and narrower. The latter objections also apply to *L. nigra* Ag. and *L. semiplena* J. Ag., to which it shows closest relationship.

HYDROCOLEUS RAVENELLII Wolle, Bull. Torr. Bot. Club **6**:183. 1877.

Trichomes 10–16 μ wide, on the average "ca. 12 μ." Sheaths not staining blue or violet with chlor-zinc-iodine, up to 8 μ wide. Fig. 6.

WOLLE's typical station is "pasture grounds, Houston, Texas"; our Missouri specimens develop in thick red crusts on soil after rainy seasons. GEITLER (**4**) cites the alga as growing "in stehenden Wasser."

Previously reported (**2**) from Boone Co., where it had been found on moist soil in the Perche Creek bottoms near Midway. Sept. 28, 1930, *Drouet 690*. Lately picked up "in an oak glade, soil dry and acid," near Pacific, Franklin Co., Nov. 20, 1932, *Ward Sharp*. Also on soil in a low meadow by Hinkson Creek south of Columbia, Boone Co., Apr. 8, 1933, *Drouet 1080*.

Scytonemataceae

PLECTONEMA BORYANUM Gomont, Bull. Soc. Bot. Fr. **46**:36. 1899.

Trichomes 1.3–2 μ diam. Sheaths not staining blue or violet with chlor-zinc-iodine. Fig. 7.

Appearing in the bottom of a culture dish in the laboratory at Columbia, Sept. 26, 1928, *Drouet 218*. To this species may be referred also a specimen marked "*Plectonema! boryanum?* Gomont. On pots, greenhouse," Missouri Botanical Garden, St. Louis, Mar. 24, 1918, *F. S. Collins 24*, in Herb. Mo. Bot. Gard. The material is not in an excellent state of preservation, so that I have not been able to make out the branching of the filaments. The habit of the trichomes otherwise appears to correspond with that of my material.

PLECTONEMA NOTATUM Schmidle var. **saegeri,** var. nov.

Var. cum trichomatibus 1.5–2.0 μ latis, indistincte articulatis. Hab. in aquariis, Columbia, Missouri.

Plant mass pale green or blue-green, laminated, floating or subaquatic; filaments parallel or entangled, long; false branching seldom, mostly in pairs; trichomes 1.5–2.0 μ wide, not constricted, with indistinct cross walls; cells half as long as wide to quadrate, seldom longer; cell contents pale blue-green, homogeneous; sheaths conspicuous, thin, colorless, not staining blue with chlor-zinc-iodine. Fig. 8.

In dilute Knop's solution with species of Lemnaceae grown by Dr. A. C. SAEGER, Columbia, Dec. 30, 1932, *Drouet 1050, 1051*. The filaments were especially abundant and well developed in the solution in which salts of manganese were absent and were less abundant in the solution containing such salts. In 3 per cent aqueous formalin (40% formaldehyde) the trichomes exhibit clearly the length of the cells, for by this treatment the walls become conspicuous and small granules are produced within the protoplasm.

PLECTONEMA TOMASINIANUM var. GRACILE Hansg. *in* GEITLER, Cyanophyceae (Blaualgen), 689. 1932.

Trichomes 12–13 μ diam. Sheaths staining blue with chlor-zinc-iodine. Fig. 9.

Reported previously (3). In Meramec River at Mincke, Franklin Co., and in the large lake at Lake Hill, St. Louis Co., Aug. 17 and 24, 1929, *Frances Holtzwart.* On mosses and rocks, Maramec Spring, Phelps Co , Nov. 1, 1931, and Mar. 21, 1932, *Drouet 950, 973.*

SCYTONEMA AUSTINII Wood, Smiths. Contrib. Knowl. **19**:58. 1872.

Trichomes 4–10 μ diam. Sheaths not staining blue or violet with chlor-zinc-iodine. Fig. 10.

Bluffs of the Osage River, Warsaw, Benton Co., Aug. 26, 1897, *Wm. Trelease*, in Herb. Mo. Bot. Gard.

SCYTONEMA HOFMANNI Agardh, *in* GEITLER, Cyanophyceae (Blaualgen). 772. 1932.

Trichomes 5–6 μ wide. Sheaths not staining blue or violet with chlor-zinc-iodine. Fig. 11.

"Big Spring, Current River, Carter Co." Likely on damp cliffs, Sept. 10, 1897, *Wm. Trelease*, in Herb. Mo. Bot. Gard. Reported by DR. HENRI HUS (5) and by MOORE and KARRER (6) from the Missouri Botanical Garden, St. Louis.

SCYTONEMA MIRABILE (Dillw.) Bornet, *in* GEITLER, Cyanophyceae (Blaualgen), 775. 1932. *S. figuratum* Ag.

Filaments 15–21 μ diam., trichomes (4–)6–10 μ diam. Sheaths not staining blue or violet with chlor-zinc-iodine. Fig. 12.

Cliffs at Big Spring, Carter Co., Sept. 10, 1897, *Wm. Trelease*, in Herb. Mo. Bot. Gard. Reported previously (2) from Montgomery Co., on a cliff at Bigsprings, Nov. 9, 1930, *Drouet 726.*

I wish to acknowledge the courteous cooperation of the members of the Department of Botany of the University of Missouri and of Dr. J. M. GREENMAN of the Missouri Botanical Garden.

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X-RAY DOSAGE IN RELATION TO GERMINATION OF PECAN NUTS

H. P. TRAUB AND H. J. MULLER[1]

(WITH ONE FIGURE)

As a part of the project in pecan breeding, the attempt is being made to induce mutations by means of short wave length radiation (2). Such a procedure appears justified since it has recently been shown that not all mutations induced by this means in living organisms are losses (4). When these experiments were undertaken, no workable technique for the control of pollination in the pecan had been reported (6). This made it necessary to postpone work on the irradiation of pecan pollen prior to its use in breeding experiments.

Results by various workers have shown that the number of mutations produced by short wave irradiation is a function of the total energy of ionization involved, that is, the number of electrons released and the speed and distance they travel, regardless of the source of the electrons (1, 3, 5). The initial experiments here reported are therefore logically concerned with the irradiation of pecan nuts (embryos) prior to planting, as a first step in determining the maximum possible x-ray dosage with still a fair percentage of germination.

Two varieties were employed in the experiment, Halbert and Payne. The particular lots included are described in table I. The Halbert lots were grown in three different localities.

The treatments were given with Victor Company broad-focus Coolidge tube with tungsten target and Snook transformer, at 50 kilovolts peak and 10 milliamperes, through a screen of aluminum 1 mm. thick, at a distance of 14 cm. from the target. The length of treatment ranged from none (check) to 160 minutes, beginning with 10 minutes and proceeding by geometrical progression to 160 minutes.

[1] The senior writer is now engaged in subtropical fruit production research and Dr. C. L. SMITH is continuing, in cooperation with Dr. H. J. MULLER, the work reported in this paper.

On April 16, 1931, the treated nuts and checks were treated in a dilute solution of $CuSO_4$ and then stored at 32°–34° F. in an electric refrigerator until May 4, when the nuts were stratified in sand and allowed to sprout in a cold frame. The results are presented in table II.

TABLE I

DESCRIPTION OF FOUR LOTS OF PECAN NUTS USED IN THE EXPERIMENT; CROP OF 1930

Variety	Where grown	Total weight (gm.)	No. of nuts per lb.	Percentage kernel	Shell thickness		Remarks
					mm.	Percentage difference over Payne	
		Shell thickness 0.87–0.95 mm.					
Halbert no. 43	Southwest Texas	5.8	77	56	0.87	17	Grown on bottom land
Halbert no. 2	Central Oklahoma	5.8	78	57	0.87	17	Grown on bottom land
Halbert no. 170	Northwest Texas	6.5	69	54	0.95	10	Land naturally sub-irrigated
		Shell thickness 1.06 mm.					
Payne no. 183	Arizona	5.9	76	54	1.06	.....	Land irrigated

The three lots of Halbert nuts showed marked variation in germinating power, 50, 70, and 100 per cent, as indicated by the check treatments (table II). In the case of the Payne nuts, the germination for the 20 minute treatment was as high (80%) as the check treatment, and the results for the 10 minute treatment were lower (65%) than the check. Germinating power apparently has not been accurately established on the basis of the small samples used. There appears to be a consistent decrease in percentage germination, however, due to increase in dosage after the 20 minute treatment in all cases: 70% to 0%, 37% to 0%, 40% to 0%, and 80% to 3% as

shown in table II. No Halbert nuts sprouted at 80 minutes' exposure; 23% Payne nuts germinated after treatment for 80 minutes, and 3% of the same variety after 160 minutes' treatment.

TABLE II

EFFECT OF VARIOUS DEGREES OF EXPOSURE TO X-RAYS ON GERMINATION OF PECAN NUTS; 1931

Variety	Length of treatment (min.)	Number of nuts	Number germinated on						Percentage germinated 8/27	Remarks
			5/20	5/27	6/10	6/27	7/11	8/27		
		Shell thickness 0.87–0.95 mm.								
Halbert no. 43	None	10	1	2	5	5	5	7	70	1 died
	10	10	.....	.....	3	4	4	7	70	2 died
	20	14	.....	.....	5	5	5	6	42	.....
	40	16	.....	.....	2	3	4	5	31	.....
	80	10	.....	.....	.....	.....	.....	.....	0	.....
	160	10	.....	.....	.....	.....	.....	.....	0	.....
Halbert no. 2	None	16	.....	.....	1	6	6	8	50	1 died
	10	16	.....	.....	1	5	5	6	37	1 died
	20	16	.....	.....	.....	.....	.....	6	37	.....
	40	12	.....	.....	.....	.....	.....	.....	0	.....
	80	16	.....	.....	.....	.....	.....	.....	0	.....
	160	14	.....	.....	.....	.....	.....	.....	0	.....
Halbert no. 170	None	10	1	3	9	10	10	10	100	2 died
	10	11	.....	.....	3	4	5	6	54	.....
	20	10	.....	.....	3	3	3	4	40	1 died
	40	11	.....	.....	1	1	1	4	36	1 died
	80	11	.....	.....	.....	.....	.....	.....	0	.....
	160	11	.....	.....	.....	.....	.....	.....	0	.....
		Shell thickness 1.06 mm.								
Payne no. 183	None	30	.....	.....	10	20	23	24	80	.....
	10	32	.....	1	9	12	13	21	65	.....
	20	31	.....	2	7	18	20	25	80	.....
	40	30	.....	.....	4	6	11	12	40	.....
	80	30	.....	.....	2	2	6	7	23	1 died
	160	30	.....	.....	.....	.....	.....	1	3	.....

The plants in the case of the higher exposures were slower in sprouting, as shown in table II and figure 1. Later some of these late arrivals caught up, and in some cases even surpassed the checks.

Some of the embryos from treated nuts showed marked multiple sprouting and some plants remained dwarf and weak. It is of interest to note that, in many cases of longer exposure, the seed germ was killed but the cotyledons were partially or not at all damaged and functioned as living tissue. In a number of cases the tap root developed normally but no above-ground sprouts appeared (fig. 1.)

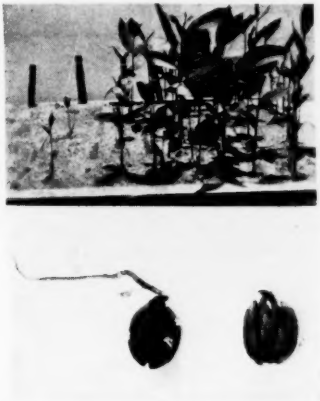

FIG. 1.—Upper: Payne seedlings, June 24, showing delayed germination for higher treatment. Lower: individual with root development only (on left); individual with seed germ destroyed (black) but cotyledons are growing and bursting nut (on right).

On the basis of these preliminary results, it appears that the maximum dosage with a reasonable percentage of germination, 25%, probably lies somewhere between 40 and 80 minutes' exposure under the conditions of the experiment.

It is shown in table I that the difference in shell thickness of Halbert lots as compared with the Payne lot ranged from 10 to 17% less for the former lots. In view of the relatively small numbers used, however, it would be hazardous to attempt to explain the difference in results on the basis of shell thickness. In future experiments the effect of this factor should be given some attention.

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CURRENT LITERATURE

The prairie

This comprehensive study is the third in a series of investigations on the prairies by WEAVER and his collaborators, HIMMEL and FITZPATRICK.[1] The previous works[2] dealt with the environment and the ecology of the dominant grasses. The present paper is concerned with the structure and dynamics of the vegetation and the ecology of the secondary grasses and forbs. The three constitute a very complete survey of the field.

The plan for the survey was to cover a representative area of large size. The region investigated measures about 300 miles north-south and 200 miles east-west; it includes portions of Kansas, Nebraska, Missouri, Iowa, South Dakota, and Minnesota. This is the heart of the "tall-grass prairie." One hundred thirty-five representative areas were studied, 20–360 acres in extent, distributed as uniformly as possible over the region.

Six principal types are recognized:

Upland types—

- Big bluestem consociation (*Andropogon furcatus*)
- Slough grass consocies (*Spartina michauxiana*)
- Tall panic grass-wild rye consocies (*Panicum virgatum, Elymus canadensis*)

Lowland types—

- Little bluestem consociation (*Andropogon scoparius*)
- Needle grass consociation (*Stipa spartea*)
- Prairie dropseed consociation (*Sporobolus heterolepis*)

These are first described as units. Detailed analysis follows, based upon very numerous quadrats. Distribution of major grasses within the consociations, analysis of the vegetation in the various types, relative height of grasses in different parts of the region are presented. In a previous paper (WEAVER and FITZPATRICK, *l.c.*) the ecology of the major grasses has been thoroughly treated; therefore, in this, particular emphasis is laid upon the minor grasses and especially the forbs. Upland and lowland species are considered separately, and under each of these headings there is further subdivision into a small group of important

[1] WEAVER, J. E., and FITZPATRICK, T. J., The prairie. Ecological Monographs **4**: 109–295. 1934.

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WEAVER, J. E., and FITZPATRICK, T. J., Ecology and relative importance of the dominants of tall-grass prairie. BOT. GAZ. **93**: 113–150. 1932.

species and a larger one of those of lesser import. For each species there is very complete discussion of habit, ecological characteristics of root and shoot, life history, seasonal régime. The detailed study of individual species is followed by discussion of seasonal aspects, height growth and physiological activity, contacts with other major vegetation types, and invasion and succession.

In style, the work shows a happy combination of the scientific and the popular mode of presentation. It is pleasant reading and at the same time nothing of accuracy is lost. The aesthetic aspects of the prairie receive their full share of recognition. A point particularly worthy of commendation is the following through of complete seasonal development, both with respect to individuals and to communities. Even the winter aspect is not neglected. This has very seldom been adequately done for any vegetation type; it is possible only for a student living in intimate contact with the vegetation concerned year after year.

The paper is abundantly illustrated with excellent photographs, including typical views of communities and portraits of individual plants. In many cases, forbs were lifted with masses of accompanying grasses and photographed in the laboratory. This method gives an excellent individual portrait and at the same time shows the relation of the forb to the surrounding grasses.

We are indeed fortunate in having so complete and trustworthy a survey of this great vegetation type, which in its natural state seems doomed to inevitable extinction.—WM. S. COOPER.

A chapter of botanical history

The life of an outstanding scientist is not only interesting as a personal biography but his activities and contacts with fellow workers give a cross-section through a period of his science. This is especially true when the life is long and full of accomplishments. Such is the case with Professor GOTTLIEB HABERLANDT, whose memoirs[3] not only show the development of physiological plant anatomy but also give a survey of botanical history from NÄGELI, SCHWENDENER, DE BARY, SACHS, STRASBURGER, PFEFFER, and WIESNER to our day. There are primarily three great contributions which HABERLANDT gave to botany besides many minor ones: his handbook of physiological plant anatomy; his studies of the sense organs of plants; and his ecologic, physiologic, and organographic treatment of the tropical vegetation, a result of his visit to the botanical garden at Buitenzorg in Java. During his span of life, which will reach eighty years by November 28, 1934, HABERLANDT has seen the development of plant physiology as an independent branch in teaching botany. He himself organized an "Institut" for plant physiology and anatomy in Graz, Austria, and another one in Berlin. These institutes have their independent greenhouses and gardens, in spite of the existence of other large botanical gardens and buildings in both places.—A. C. NOÉ.

[3] HABERLANDT, G., Bekenntnisse und Beobachtungen. pp. 243. Julius Springer, Berlin. 1933.

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